STUDIES ON THE L-AMINO ACID OXIDASE OF *NEUROSPORA CRASSA*

Thesis by

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Abstract

The L-amino acid oxidase of *Neurospora* is a general amino acid oxidase attacking a wide range of L-amino acids at different rates. Activity of the enzyme is dependent on substrate concentration, oxygen tension and pH. Different amino acids show different pH optima. Activity is significantly reduced by excess substrate, and competition is exhibited between mixed substrates.

L-oxidase production by mycelium is increased 4 to 10 fold by biotin limitation. This effect is not produced by: changes in extractability of the enzyme; reduced level of growth; defective ammonia, aspartic acid, or riboflavin metabolism; or production of an inhibitor of the enzyme.

The enzyme is adaptively formed during growth in the presence of substrate amino acids. Factors affecting the degree of adaptation are biotin and substrate concentrations, pH and strain differences. Deadaptation is produced by excess biotin or the removal of substrate. The relation of adaptation to the low biotin effect is discussed.

The L-oxidase is involved in detoxification of canavanine. Strain differences in canavanine sensitivity are paralleled by certain qualitative differences in L-oxidase activity. Canavanine resistance is increased on low biotin. The relation of these observations to canavanine sensitivity and its genetic control is discussed.

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I. Introduction

A large number of the mutants of Neurospora which have been extensively studied are deficient in synthesis of amino acids. The function of the L-amino acid oxidase in general amino acid and nitrogen metabolism is unknown. A general survey of the properties and conditions of occurrence of the enzyme may throw some light on this question.

Work on the L-oxidase was undertaken with two objects in mind, both based on observations published in the first report of the occurrence of the enzyme (1). It was noted that the L-oxidase could not be detected in extracts of all wild type strains tested. This suggestion of genetic inability to form the enzyme was of special interest, since it offered the possibility of another opportunity for studying gene-enzyme relationships. The second observation, that the enzyme could be detected in extracts and in the medium only when the mold was grown on a lower than normal concentration of biotin (0.25γ instead of 5.0γ per liter) posed an interesting biochemical question. Biotin is the only growth factor required by wild type Neurospora, and little is

known of its function in the mold. Investigation of the relation of biotin to L-oxidase formation might help to fill this gap.

During the work on strain differences in L-oxidase production and on the "biotin effect", two additional problems of interest arose. The apparent adaptive formation of the L-oxidase under certain conditions was worthy of detailed consideration. The formation of an adaptive enzyme in a genetically useful organism would be of value if the potentiality of adaptation were shown to be genetically controlled. The final project undertaken was an investigation of the possible role of the L-oxidase in the resistance to canavanine observed in certain wild type strains (2). The results of these studies and of subsequent investigation of various properties of the enzyme are presented.

Several papers on the L-oxidase of Neurospora have appeared or been prepared since the initial report by Bender, Krebs, and Horowitz (1). Bender and Krebs studied the substrate range of the enzyme using 38 synthetic amino acids, and showed it to be a general L-amino acid oxidase of wide specificity (3). In this paper, comparison was made with the D-amino acid oxidase of

Neurospora (described in 1944 by Horowitz (4)), the L-amino acid oxidase of cobra venom (5), and the D-amino acid oxidase of sheep kidney. These comparisons and the general subject of amino acid oxidases were reviewed recently by Krebs (6), as well as in an earlier review (7). Two papers on the L-oxidase of Neurospora are in press at this writing. One, by the present author and Dr. N. H. Horowitz (8), presents some of the findings discussed in this thesis. Dr. K. Burton has kindly made available the manuscript of a similar paper on work done in Krebs' laboratory (9), in which many of the present findings are corroborated, and in some respects extended. Separate points of difference will be noted in the pertinent sections.

II. Materials and Methods

Strains Used

The standard strains of Neurospora listed in Table I have been used in this work. Other cultures have included ascospore re-isolates from crosses of 25a x 4A, and of 4A x 25R11. The progeny of the first crosses have been designated 25R1, 4R1, etc., to denote which parent they most resemble in canavanine sensitivity. Cultures from the second cross are all designated 11R1, 11R2, etc., regardless of sensitivity. Crosses were made on Westergaard's medium (10) or on Bacto corn meal agar.

Table I

Neurospora Strains Used

Cultures maintained on agar slants of complete medium (11), except C83, which was on minimal medium supplemented with tryptophane.

Strain	Requirement	Furnished by
4A	Wild type	Dr. N. H. Horowitz
25a	"	"
1A	"	"
12a	"	"
5297a	"	"
5256A	"	"
Lindegren-a	"	"
Sh-Abbot-A	"	Dr. H. A. Krebs
Sh-Abbot-a	"	"
Sh-5256A	"	"
Sh-5297a	"	"
Sh-Lindegren-A	"	"
CMI 3411	"	Dr. K. Burton
44105-S	Threonine, etc.	Dr. H. Garner
44105-R	"	"
37101	Lysine	Mr. N. Good
C-83A	Tryptophane	Dr. F. Haskins
32213	Amino nitrogen	Mrs. M. Mitchell
68604	Methionine	Mr. H. Gershowitz
85518	Thiosulfate	Dr. N. H. Horowitz

Experimental Cultures

Growth experiments were carried out with 125 ml. Erlenmeyer flasks containing 20 ml. of liquid medium. Growth was measured by weighing pads after drying at 90°. Cultures to be used for the preparation of extracts were grown for 5-15 days in Fernbach flasks with 200-500 ml. of medium, or in 750 ml. Erlenmeyer flasks with 200 ml. medium. To obtain enzyme from the medium, the mold was grown for at least 21 days in 200 ml. medium in Fernbach flasks. All media were sterilized by autoclaving at 15 lbs. for 10 minutes. Experimental cultures were grown at 25°. Minimal and nitrogen-free minimal medium (K for NH_4 tartrate; NH_4NO_3 omitted) were used. (See Appendix).

Enzyme Preparations

L-amino acid oxidase activity has been studied in crude or partially purified cell-free extracts of mycelium, in the insoluble residue remaining after extraction, and in the material precipitated from medium with ammonium sulfate. In preparing extracts, the general procedure was that described by Horowitz (4), with variations as noted for the specific experiments. The mycelium was washed on a Buchner funnel and suction flask, then ground with sand and M/60 pyrophosphate buffer (pH 8.5) in a chilled mortar. The homogenate was centrifuged to remove sand and cell debris and 0.25 volume of M/5 buffer added to the supernatant. In most cases 2 ml. of M/60

buffer was used per gram fresh weight of mycelium. Such preparations will be called "crude extracts". For many of the experiments reported the protein of the supernatant was precipitated by the addition (in an ice bath) of solid or saturated $(NH_4)_2SO_4$ to give 0.8 saturation. After centrifugation the precipitate was redissolved in buffer and used as such, or dialyzed. Crude extracts or redissolved salt precipitates were dialyzed against M/60 phosphate buffer (pH 6), usually overnight, and in the cold. Before use, 0.25 volume of M/5 buffer was added to dialyzed preparations.

In a few cases, the insoluble material from the initial centrifugation of ground mycelium was used in determinations of enzymatic activity. It was prepared for use by washing in buffer on the centrifuge, the sand being removed by settling and decanting. The final, washed, sand-free precipitate was suspended in buffer and mixed well when used.

L-oxidase activity in culture medium can be determined on unconcentrated medium after filtration and buffering by the addition of 0.25 volume of M/5 buffer. The low activities of these dilute preparations made it necessary to concentrate the enzyme for most purposes. The clear, amber, filtered medium was chilled in an ice bath and solid $(NH_4)_2SO_4$ added to 0.8 saturation. The precipitate, containing the enzyme, was thrown down in the centrifuge and was resuspended in water (5 ml.

for each 300 ml. of the original filtrate). Such preparations were used directly or after dialysis against phosphate (as with extracts). Concentrated enzyme preparations were stored at -20°. Undialyzed preparations retained high activity for several months. After dialysis a noticeable decrease of activity occurred within a few weeks. This difference may be due to stabilization by $(NH_4)_2SO_4$, or to inactivation by phosphate (12).

An enzyme powder has been prepared by freeze-drying. Precipitated preparations from numerous lots of medium collected over a period of months were pooled and dialyzed before lyophilization. The resulting powder retained all the activity (on a dry weight basis) of the original solution. Stability should be comparable to that of acetone-precipitated and vacuum dried enzyme (9), which is said to lose no activity in six months at room temperature, in vacuo over H_2SO_4 and solid NaOH. However, the catalase originally present was found to have largely deteriorated after two months, necessitating the use of added catalase to produce stoichiometric oxygen uptake (see below).

Determinations of Enzyme Activity

Quantitative determinations of enzyme activity were made in the Warburg apparatus at 30°. Vessels of 15-20 ml. total volume were used with a reaction volume of 2.25 or 2.50 ml. Alkali was added to the center well (0.2 ml. of 2N NaOH). The gas phase was oxygen, the

vessels being closed after running oxygen through for two minutes. After gassing, a 10 minute period was allowed for thermal equilibration. When extracts were used, one or more readings were made before mixing. This was not necessary with enzyme from the medium which has no endogenous respiration.

Preliminary estimates of enzyme activity were sometimes made by measuring keto-acid production by the colorimetric 2, 4-dinitrophenylhydrazine method of Friedemann and Haugen (13). Color of the final alkaline solution was estimated visually or measured on the Klett colorimeter.

Enzyme activity has been expressed variously as μl. O_2/hour/g. fresh mycelium, μl. O_2/hour/mg. dry mycelium and μl. O_2/hour/mg. protein. The "fresh weight equivalent" (FWE) in g./ml. of extracts is taken as the ratio of the original fresh weight (after washing and drying on the Buchner funnel) to the final volume of extract as used. This is a somewhat inaccurate measure for purposes of comparison for two reasons. First, as a true measure of amount of mycelium, the fresh weight is unreliable. For example, the dry weight of a number of mycelial pads prepared by the same procedure has been from 14 to 37% of the fresh weight (mean 22±5). In cases where the total yield of enzyme from a whole pad is calculated, this error is cancelled out. Another cause of error is the difficulty of obtaining in the final extract

all the enzyme present in the original pad. Mechanical losses occur in the original precipitate, and on the containers used. Efforts have been made to minimize such losses, which are particularly significant with small pads. Comparison of activities on a wet weight basis is thus significant only for large differences, and for total yield of enzyme.

The use of dry weight as the basis of enzyme activity eliminates the first objection. The dry weight/wet weight ratio of a pad has been determined by weighing a small piece of the washed mycelial mat before and after drying at 90°. The "dry weight equivalent" (DWE) in mg./ml. is then calculated by dividing the total dry weight of the mycelium extracted (=wet weight x $\frac{\%\text{ dry weight}}{100}$ - weight of piece dried) by the final volume of extract. Activities expressed on this basis are considered to be sufficiently accurate for the work reported here.

The third expression of activity based on protein content is most valuable for enzyme precipitated from culture medium. Protein was determined gravimetrically by the following steps: the solutions to be tested were transferred quantitatively to tubes, the pH adjusted to 6, tubes immersed for three minutes in boiling water, chilled, centrifuged, and the precipitate washed with water on the centrifuge and dried in tared beakers. Protein in extracts measured by this method ranged from

4.5 to 14.5% of the dry weight equivalent with a mean of 9.0±1.8% (33 determinations). Activities of low biotin extracts expressed on this basis with leucine as substrate ranged from 1.8 to 5.6 µl./hour/mg. with values for extracts of adapted cultures going as high as 44.8 (leucine as substrate, pH 8). Enzyme from medium gave values from 245 to 375 with a mean of 320, (leucine, pH 6), which can be extrapolated to give a mean Q_{O_2} of 720 at pH 8.

Chemical Preparations

L-α,γ-diamino-butyric acid was prepared from L-glutamic acid by the method of Adamson (14). Six grams of NaN_3 (incomplete amount) were added to 14.7 g. L-glutamic acid in 30 ml. $CHCl_3$ and 50 ml. concentrated H_2SO_4. After cooling, neutralization to pH 3 with $Ba(OH)_2$ solution, filtration, and the addition to the filtrate of 33 g. picric acid in 700 ml. water, there was obtained 17.8 g. of L-α,γ-diamino-butyric acid dipicrate (31% yield; melting point, 176.5-178.5°). The dipicrate was dissolved in 190 ml. hot water and 100 ml. concentrated HCl added. After removal of picric acid by filtration, the filtrate was extracted with ether and ethyl acetate and concentrated over boiling water. The final precipitate was washed with absolute ethanol and ether and dried in vacuo. The white, crystalline product was not recrystallized, and appeared chromatographically pure.

Final product: L- α,γ -diaminobutyric acid · 2HCl; 4.6 g. (24% overall yield)

Melting point:

Observed	205-6° (dec.)	(Capillary tube)
Literature	195-6° (dec.)	(14)
	202-4° (dec.)	(15)

Nitrogen content:

Found	14.95%*
Calculated	14.65%

Casein hydrolysate was prepared by 19-21 hour hydrolysis with HCl. Two lots were prepared from 112 and 200 gms. of Nutritional Biochemical Co. "Vitamin-test" casein. The casein was dissolved in 400 ml. water and 450 ml. concentrated HCl per 100 g. The solution was refluxed over a gas flame discontinuously for about 20 hours. The hydrolysate was concentrated to a syrup in vacuo at a temperature below 60° and reconcentrated after the addition of 300 ml. water per 100 g. casein. The final syrup was diluted with water to 400 ml. per 100 g. casein and the pH adjusted to 3 with solid NaOH. The solution was decolorized by two treatments with Norite (25g./100g. casein; 15 minutes stirring). The clear, colorless filtrate was diluted to give a 10% solution (based on original casein content), dispensed into screw-top 50 and 100 ml. bottles and autoclaved. When tested in biotin-free medium at concentrations

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* Analysis by Mr. G. A. Swinehart

from 0.5 to 2.5%, these preparations showed no biotin activity for growth of wild type 4A, in contrast to the apparent biotin activity of the casein hydrolysate used by Burton (9).

All amino acids used were from commercial sources, except diamino-butyric acid and the following compounds furnished through the courtesy of the persons noted:

L-Canavanine	Dr. N. H. Horowitz
L-Cystathionine	Dr. N. H. Horowitz
L- α-Amino-adipic acid	Mr. N. Good
DL- α-Amino-ϵ-hydroxy-caproic acid	Mr. N. Good
DL- α-Amino-γ-hydroxy-butyric acid	Dr. M. Fling

III. Properties of the Enzyme and Kinetic Studies

Reaction and Stoichiometry

The enzyme has been shown to be a typical oxidative amino acid deaminase (7). Using L-alanine as substrate, simultaneous determinations of oxygen consumption and of ammonia and pyruvate production were made. Ammonia was determined by nesslerization after distillation by the Conway-Byrne technique. Pyruvate was measured by the "direct method" of Friedemann and Haugen (13). The results are shown in Table II. In the overall reaction one atom of oxygen is consumed and one molecule each of ammonia and pyruvate are produced per molecule of alanine oxidized. As noted previously (1), the preparations contain catalase, so that hydrogen peroxide does not

Table II

Oxidation of L-alanine--Stoichiometric Relations

Each vessel contained 0.25 ml. M/30 L-alanine, 0.5 ml. dialyzed enzyme, and 1.5 ml. M/15 phosphate buffer, pH 6.

Vessel	O_2 consumed		Pyruvate produced			Ammonia produced		
	µl.	µM	γ	µM	µM/µM O_2	γ N	µM	µM/µM O_2
1	83	3.7	680	7.7	2.08	122	8.7	2.35
2	92	4.1	750	8.5	2.07	118	8.4	2.04
3	91	4.1	705	8.0	1.95	114	8.1	1.97
Mean					2.03			2.12

accumulate. That peroxide is produced, however, is indicated by the fact that the addition of ethanol to the reaction mixture causes an increase in the oxygen uptake (16). In two such experiments, with L-leucine as substrate, the oxygen uptake was respectively 1.33 and 1.64 atoms per molecule oxidized. No oxidation of ethanol occurred in the absence of an amino acid substrate. Further evidence for the role of catalase was obtained with the use of lyophilized enzyme. Without the addition of catalase, oxygen uptake catalyzed by this preparation was 1.5 atoms per mole of substrate. When catalase* was added, oxygen uptake was 1.0 atom per mole. Deamination of canavanine was also shown to produce one mole of ammonia per atom of oxygen consumed.

* Crystalline, Beef-liver Catalase, Worthington Biochemical Laboratory

It is concluded that the reaction catalyzed by the Neurospora L-oxidase is that formulated for other amino acid oxidases (6). The primary enzymatic reaction,

$$R - CHNH_2 - COOH + O_2 \longrightarrow R - C{:}NH - COOH + H_2O_2,$$

is followed by a spontaneous decomposition of the imino-acid to the keto-acid and ammonia,

$$R - C{:}NH - COOH + H_2O \longrightarrow R - CO - COOH + NH_3.$$

In the presence of catalase the peroxide is removed,

$$H_2O_2 \longrightarrow H_2O + 1/2O_2,$$

and, as indicated by the data, the overall reaction is:

$$R - CHNH_2 - COOH + 1/2O_2 \longrightarrow R - CO\text{-}COOH + NH_3.$$

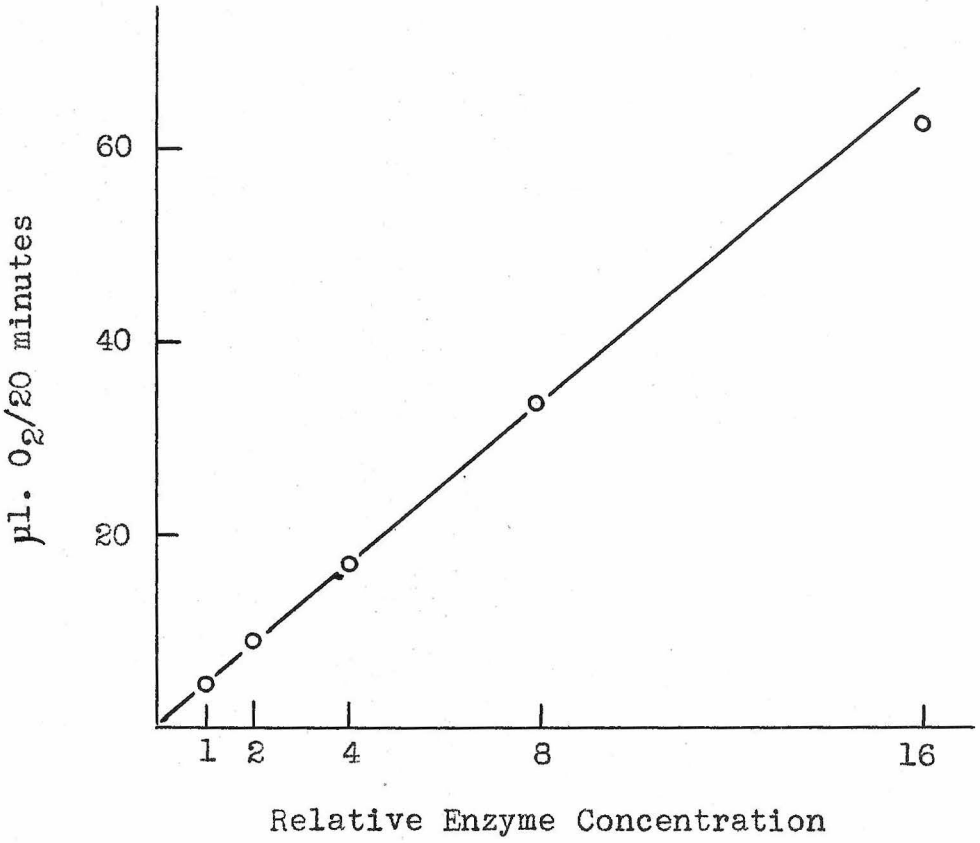
Enzyme and Substrate Concentration Effects

The initial rate of oxidation is linear with respect to enzyme concentration (Fig. 1).

When the rate of oxidation is plotted against substrate concentration, an optimum concentration is observed (Fig. 2). A similar phenomenon has been reported for the L-amino acid oxidase of snake venoms (5, 17).

Attempts to determine Michaelis constants from such data have been unsuccessful, quite discrepant values being obtained for three different experiments. Two graphical methods of calculation were used, based on Haldane's equations (18, p. 83) and the method of Lineweaver and Burk, Case III (19). Both are based on the assumption of the formation of an inactive enzyme-substrate complex by the combination of more than one substrate molecule with each enzyme molecule (or site). Determinations of sufficient accuracy require several changes in procedure. Reaction rates, especially at low substrate concentrations where oxygen uptake is small, could better be measured by colorimetric or spectrometric determination of keto-acid formation. This would also eliminate any discrepancies in oxygen exchange produced by variations in catalase activity. For the methods of Lineweaver and Burk, higher and lower concentrations of substrate should be employed. The inhibition by excess substrate is not likely to be inhibition of catalase, since that would increase rather than lower the initial

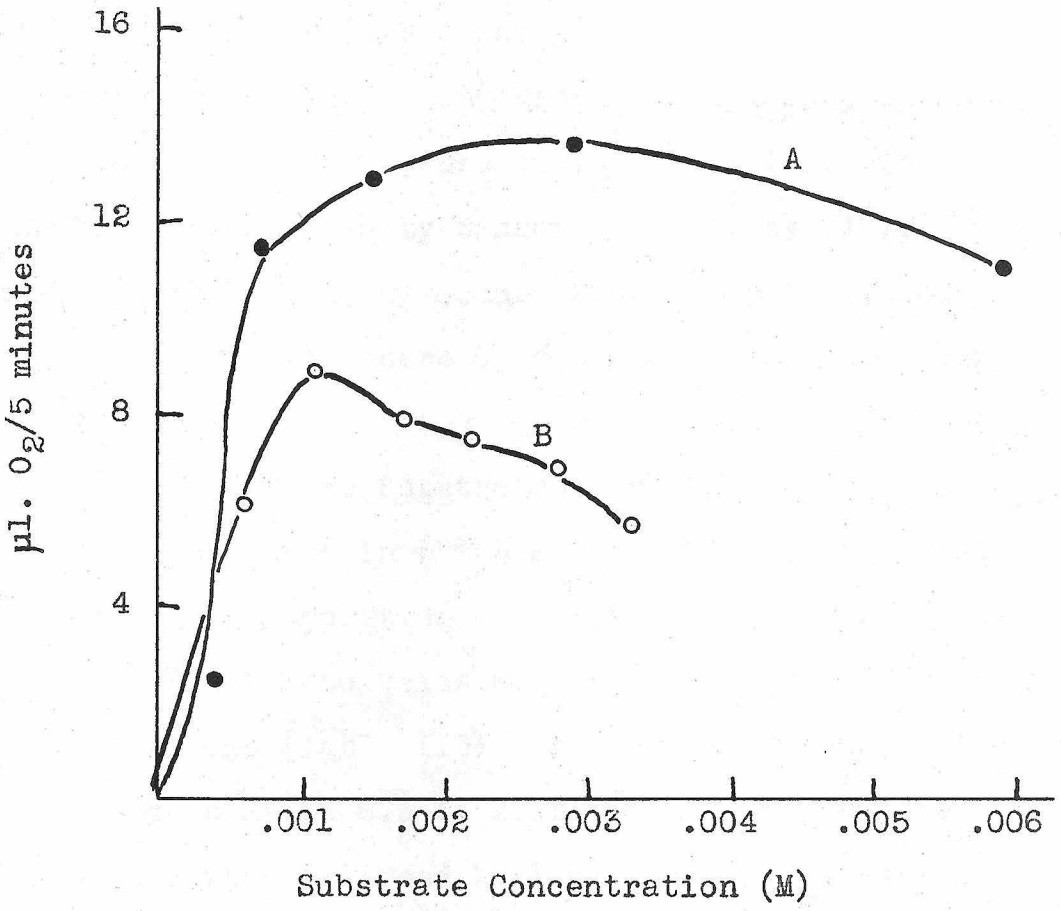
Figure 1



Enzyme Concentration Effect

Substrate, M/270 L-canavanine, pH 6. Enzyme, L-oxidase from medium.

Figure 2



Substrate Concentration Effects

Curve A, L-leucine, pH 8; curve B, L-canavanine, pH 6. Initial rates.

rates of oxygen uptake. Nor can this be a case of inhibition by product, since it is self-evident that the rate is lowest where the amount of product is least. At substrate concentrations above the optimum, an increase of rate often occurs during oxidation. The possibility suggested by Singer and Kearney (17), "that a competition may occur between substrate and O_2 for the location where O_2 combines," should be considered too.

Oxidation of Two Substrates Consecutively

Experiments in which a second lot of substrate is added after completion of oxidation of a first lot have shown that keto-acids may be inhibitory under certain conditions (Table III). The apparent inactivation of the enzyme at pH 8.5 by histidine and canavanine (especially when followed by leucine), is suggestive of inhibition by keto-acids. The absence of such an effect with leucine may indicate that keto-acids *per se* are not inhibitory, but that the presence of an additional basic group makes them so. In similar experiments with consecutive lots of canavanine and of phenylalanine (pH 8.5), phenylalanine behaved similarly to leucine. No explanation can be offered at present for the apparent activation by histidine produced at pH 6. A similar phenomenon was noted by Edlbacher and Wiss with the D-amino acid oxidase of kidney (20).

Table III

Activation and Inactivation of L-oxidase

By First Lot of Substrate

Second substrate added five minutes after completion of oxidation of first. Final concentration: first substrate, M/270; second, M/300. Gas phase, O_2. Enzyme, 0.25 ml. of dialyzed L-oxidase from medium.

pH	First Substrate	Rate µl. O_2/hour	Second Substrate	Rate µl. O_2/hour	% change
6	L-canavanine	43.7	L-canavanine	40.6	-7
	L-leucine	33.8	L-leucine	45.0	+33
	L-canavanine	42.0	"	31.9	-6*
	L-histidine	41.3	L-histidine	65.2	+58
8.5	L-canavanine	54.6	L-canavanine	39.0	-29
	L-leucine	154.6	L-leucine	151.2	-2
	L-canavanine	58.0	"	67.6	-56*
	L-histidine	138.3	L-histidine	71.6	-48

* Compared to first lot of leucine.

Substrate Competition

The Neurospora L-oxidase resembles that of snake venom (5) in showing interference between two substrates added to the enzyme simultaneously. In Table IV are presented initial oxidation rates of a number of amino acids tested singly and in pairs. It is seen that the less rapidly oxidized amino acids inhibit the oxidation of the more rapidly oxidized ones. This result is most

Table IV

Competition between Mixed Substrates

Experiment	pH	Substrate	O_2 consumed in 1st 10 minutes
			µl
1	7.6	M/300 DL-isovaline	0
		M/600 L-phenylalanine	42
		" + M/24 DL-isovaline	30
2	5.6	M/600 L-lysine	42
		" + M/24 DL-isovaline	19
3	6.0	M/540 L-arginine	31*
		M/270 "	20*
		M/540 L-lysine	30*
		M/270 "	15*
		M/540 " + M/540 L-arginine	17*
4	6.0	M/665 L-canavanine	27
		" + M/300 L-glutamate	21
		" + " L-aspartate	20
		" + " L-methionine	10
		" + " L-lysine	5

* O_2 consumed in 20 minutes

simply interpreted on the basis of competition between the substrates for the enzyme surface. It was previously demonstrated that isovaline (α-amino- α-methyl-n-butyric acid) competitively inhibits the oxidation of methionine by the D-amino acid oxidase of Neurospora (4). Competition

is also observed when amino acids are added to a crude mycelial extract containing the enzyme. This and other effects of the presence of amino acids in extracts will be discussed below. No kinetic studies on competition have been made, largely because of the complications introduced by the fact that an excess of a single substrate is inhibitory. The kinetics of the simpler case should be worked out first, since the two types of inhibition are undoubtedly related.

Oxygen Effect

An increase in enzymatic activity of 3 to 5 fold (average 4.5) is observed when the tests are carried out in oxygen as compared to air (Table V). A similar effect has been reported for the L-amino acid oxidase of moccasin venom by Kearney and Singer (17).

Table V

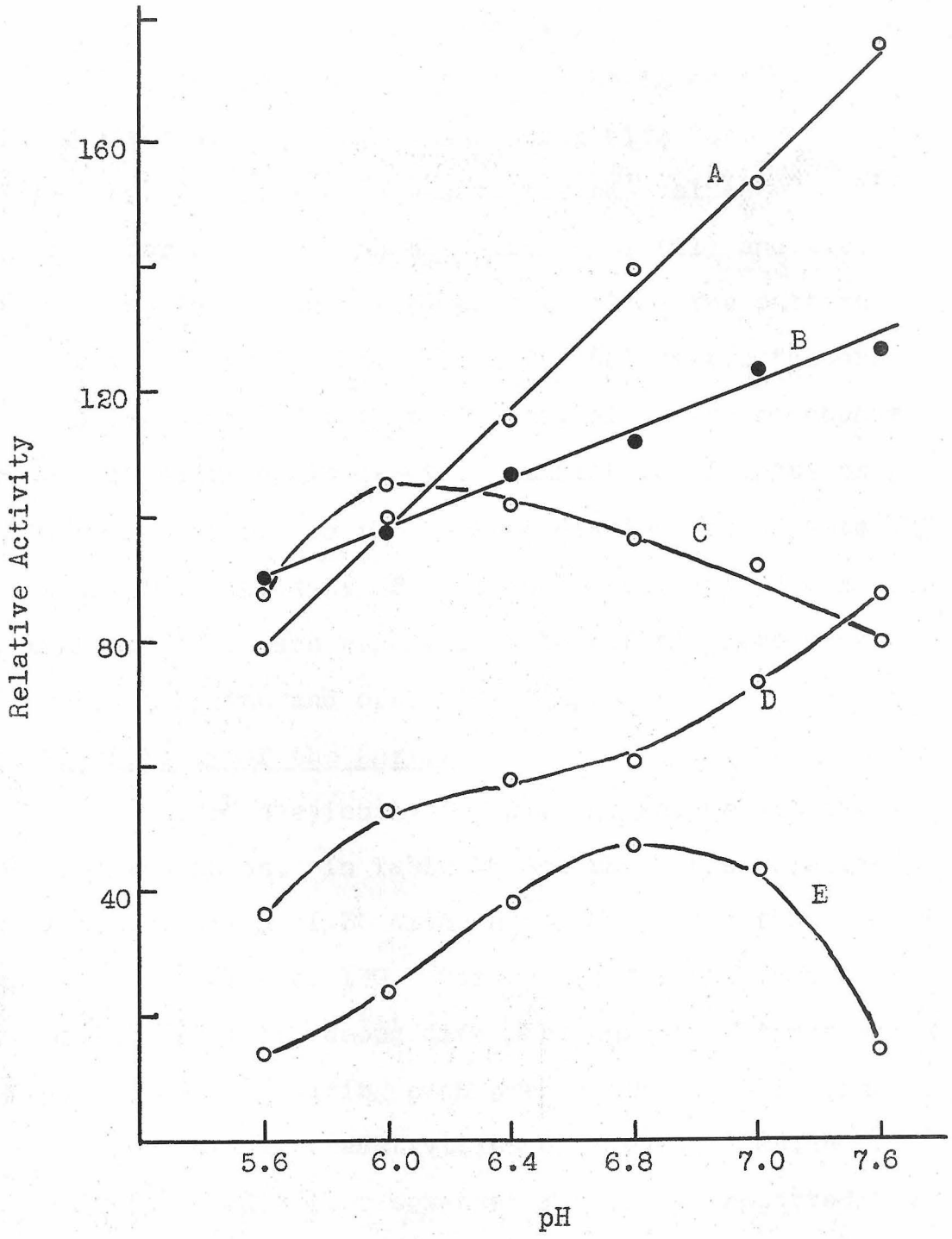
Effect of Oxygen Concentration on L-oxidase Activity

Substrate	Enzyme	Activity (μl. O_2) Air	Oxygen	Mins.	Ratio O_2/air
L-canavanine	Ppted. extract	8.2	45.2	5	5.5
"	Ppted. medium	13.4	63.2	20	4.7
"	" "	16.4	84.2	50	5.1
L-phenylalanine	" "	18.0	68.1	50	3.9
"	" "	13.8	60.6	20	4.4
L-leucine	" "	16.8	68.4	170	4.1
"	" "	9.5	38.9	170	4.1
				Mean	4.5

Effect of pH on Activity

The effect of pH on the activity of the oxidase was determined in the range pH 5.6 to pH 7.6, using phosphate buffers. Data obtained with 5 different substrates are presented in Figure 3. In the case of ornithine, the oxidation rate is only slightly affected by pH changes in the range studied, whereas with glutamate an optimum occurs at pH 6.8. Oxidation of monoamino-monocarboxylic acids is also strongly pH-dependent, with optima at pH 7.2 or higher. Esterification of the carboxyl group of histidine abolishes its reactivity (See Table VI).

These results indicate that the state of ionization of the substrate as well as the enzyme is important for activity. Additional data consistent with this idea have been obtained in subsequent experiments. A correlation can be shown between activities at different pH's and the ionization constants (pK's) of the substrates involved. The most suggestive case, based on the data of Table VI, is given by comparison of the basic amino acids, histidine, canavanine, ornithine, and arginine. The activities of these amino acids at pH 6 (140, 125, 106, and 56) are in reverse order to the basicity of the additional ionic group, of which the pK'_A values are 6.0, 7.4, 10.8, and 12.5 respectively (21, 22). This would suggest that the reactivity of a basic amino acid is optimal when the α-amino group is relatively more



Effect of pH on Activity

Curve A, L-leucine; B, L-phenylalanine; C, DL-ornithine; D, L-methionine; E, L-glutamate. Dialyzed enzyme from medium. Initial rates, expressed relative to L-leucine at pH as 100.

charged than the other basic group.

An analogous situation might be expected to hold for the dicarboxylic amino acids with respect to the ω-carboxyl group. However, the pK_2' values of 3.65 and 4.25 for aspartic and glutamic acids (21) and 4.21 for α-amino adipic acid (23) do not follow the pattern of relative activity. It is likely that steric factors play an important part in this case, since the compounds are of different chain length. Similar considerations would seem to apply to the L-α-ω-diamino acids, although the titration constants of diamino-butyric acid are not available. (They are reported to be "of the same order" as those of lysine and ornithine (24).)

Specificity of the Enzyme

As noted previously (1, 3), the enzyme attacks only L-amino acids. In Table VI are shown the relative rates of oxidation of 33 amino acids by enzyme from medium, based on L-leucine as 100. Correction for the variation in absolute activity among different enzyme preparations was made by standardizing each preparation on L-leucine.

The relative reactivities of the amino acids shown in Table VI differ somewhat from those reported by Bender and Krebs (3), particularly in the cases of histidine, lysine, and ornithine. These and other differences are undoubtedly due, for the most part, to the fact that the present values were found at pH 6, whereas the Sheffield workers made their measurements at pH 8.4,

Table VI

Rates of Oxidation of L-amino acids by Neurospora L-Oxidase

All rates are expressed relative to L-leucine as 100. L-isomer concentration M/270 in all cases except L-cystine (M/540). Oxygen uptake measured in first 10 minutes. pH 6.0.

Substrate	Relative Activity		Relative Activity
L-Histidine	140	L-Methionine	53
DL-α-Amino-n-butyric acid	134	L-Tryptophane	43
L-Canavanine	125	L-Lysine	40
L-Tyrosine	122*	DL-α-Amino-ϵ-hydroxy-n-caproic acid**	38
DL-Ornithine	106	L-α,γ-Diamino butyric acid	35
DL-Phenylalanine	106	L-Alanine	32
L-Leucine	100	L-Glutamic acid	24
L-Phenylalanine	99	L-Valine	19
DL-α-Amino adipic acid**	96	DL-Isoleucine	18
L-Cysteine	85	Glycine	0
L-Cystine	81	DL-Serine	0
DL-α-Amino-n-valeric acid	80	DL-Threonine	0
DL-Citrulline	79	L-Proline	0
L-Glutamine	64	DL-Aspartic acid	0
DL-Norleucine	61	DL-Isovaline	0
L-Cystathionine	58	L-Histidine methyl ester	0
L-Arginine	56	DL-Lactic acid	0

* Includes tyrosinase activity (see text).
** Compound furnished through the courtesy of Mr. Norman Good.

and used crude extracts. The basic amino acids are nearer their optima at the lower pH, while the reverse is true for monoaminomonocarboxylic acids.

As observed above, the reaction consumes one atom of oxygen per mole of substrate. The following exceptions to this rule were noted: tyrosine, glutamine, cystathionine, ornithine, and canavanine. (Because of their slow rates of reaction, the oxidations of glutamate, alanine, isoleucine, valine, and diamino-butyric acid were not run to completion.) Tyrosine consumes in excess of the theoretical value (> 1.8 atoms when the reaction was stopped), a fact also noted by Bender and Krebs (3). The solution turns red and then black in the course of the reaction, indicating the presence of tyrosinase in the enzyme preparation. Glutamine consumed only 0.83 atoms of oxygen, suggesting that the amide may have been partially hydrolysed to the slow-reacting acid. L-cystathionine, containing two reactive amino groups, took up 1.3 atoms instead of the expected two atoms, while canavanine and ornithine consumed, in numerous trials, 0.8-0.9 and 0.75-0.85 atoms, respectively. Incomplete oxidation (0.7 atoms) of ornithine was also obtained by Bender and Krebs. The following compounds were not attacked at a measurable rate: glycine, DL-serine, DL-threonine, L-proline, L-aspartate, DL-isovaline (α-amino-α-methyl-n-butyric acid), DL-lactate, and L-histidine methyl ester.

Physical State

The Neurospora L-amino acid oxidase is extractable and soluble in M/60 phosphate (pH 6) and M/60 pyrophosphate (pH 8-8.5), the only solutions tried. It is also soluble in culture medium, being found there after protracted growth. The site of the activity observed in the residue remaining after extraction is not definitely known. Since such residues contain unbroken cells, cell debris, nuclear material and the larger, intracellular particles, it cannot be said whether the enzyme exists in a particle bound state in the cell and is only partially solubilized during extraction, or is soluble but incompletely extracted. The necessity for devising improved methods of grinding mold mycelium is apparent. Until such methods are available, caution must be used in interpreting enzymatic data obtained with extracts in terms of the total activity of the mycelium. A method of possible application is the "fly-press" (i. e., drill press) technique of Hughes (25), which is reported to give Neurospora homogenates with "almost complete disappearance of discrete mycelia."

Extracellular Occurrence

The L-oxidase is found in culture medium in a soluble form after extended growth periods. Whether this represents active secretion or passive release into the medium can be considered only indirectly with the data at hand. Several observations point to an

autolytic mechanism, despite the objections of Krebs (6). With the strains used in the present studies, it has always been necessary to grow the cultures for nearly three weeks before any L-oxidase activity was detectable in the medium. Growth curves of strains 25a and 4A on both high and low biotin medium show rapidly increasing growth up to 3 days after inoculation with a continued, slow increase up to 9 days. After 10-11 days, a marked decline in dry weight occurs, the dry weights after 15 days being 66 to 83% of the maximum reached. Similar observations have been made previously (E. g., Horowitz, 26).

A significant amount of autolysis does not seem to occur until after 10 days growth (in 20 ml. medium in 125 ml. Erlenmeyer flasks). Some difference might be expected when the mold is grown on other volumes of media and with different area:depth ratios, although the concentration of all added constitutents is the same. The data of Burton (9) differ in that he found measurable amounts of L-oxidase in the medium at 4-5 days growth when the dry weight was probably still increasing. However, his use of strain CMI 3411, which will be seen below to be different from most others in several respects, grown in shallow medium (1 cm.) may produce the difference. That the volume-surface-depth relations affect the release of the enzyme has been suggested during the preparation of enzyme from various lots of medium. The larger the volume of medium and the greater its depth, the less

enzyme seems to be released in a comparable growth period. In very shallow medium the enzyme is found after shorter periods of growth. How closely such results correlate with the extent of autolysis has not been determined. In conclusion, it can only be said that it is possible that the L-oxidase occurs in low biotin medium as a result of autolytic action.

Stability of the Enzyme

Only incidental observations have been made on factors affecting the stability of the L-oxidase. They will be summarized briefly. The L-oxidase is stable at -20°, is not impaired by freeze-drying, ammonium sulfate or ethanol precipitation, or 24 hour dialysis. It is partially destroyed by precipitation and drying with acetone. It is inactivated by heating to 70° at pH's 6 and 8.5, even in the presence of L-leucine (3.7×10^{-3}M). Burton found that leucine (5×10^{-3}M) protects the enzyme against heat inactivation at 55° and pH 6 (9). The L-oxidase does not seem to be affected by phosphate buffer as is the similar enzyme of snake venom (12). L-oxidase precipitated from medium dissolved in water without dialysis was tested in two buffers at two pH's (Table VII). The enzyme was in the main well, i. e., dissolved in the buffer, during gassing and equilibration to maximize any possible effect. No difference was noted between activity in phosphate or maleic acid-tris (hydroxymethyl)-aminomethane (27) at pH 6, nor between

Table VII

L-oxidase Activity in Phosphate and Non-phosphate Buffers

2.0 ml. buffer, 0.25 ml. M/30 L-leucine, 0.25 ml. enzyme precipitated from medium.

Buffer	Final concentration	pH	Oxygen uptake in 15 mins. µl.
Phosphate	0.053 M.	6.0	21.4
Maleic-THAM*	0.080 M.	6.0	20.6
Pyrophosphate	0.032 M.	8.0	46.2
THAM-HCl	0.040 M.	8.0	44.3

* Tris(hydroxymethyl)-aminomethane

pyrophosphate or tris(hydroxymethyl)-aminomethane (28) at pH 8. However, as noted above, precipitated enzyme loses activity in the frozen state more rapidly after dialysis against phosphate buffer (pH 6).

Prosthetic Group

No extended attempts to identify the prosthetic group of the L-amino acid oxidase were made by the present author. It was found, however, that the acid-ammonium sulfate method employed by Warburg and Christian for resolution of kidney D-amino acid oxidase (29) destroyed the activity of the Neurospora L-oxidase. Reconstitution was not effected by the addition of boiled enzyme. Burton presents conclusive evidence that flavin-adenine-dinucleotide is the prosthetic group (9).

IV. The Mechanism of The Biotin Effect

The Effect of Low Biotin

When an L-amino acid is added to a crude extract of *Neurospora* mycelium grown on medium containing biotin at 0.25γ/liter (1/20 of the amount normally used), an increase of oxygen uptake is observed. The total increase, if incubation is continued, represents the uptake of one atom of oxygen per mole of L-amino acid added. With a crude extract from mycelium grown on the standard concentration of biotin (5.0γ/liter), no increased uptake is noted, even after prolonged incubation (Table VIII). The increased uptake by low biotin extracts was shown (1) to be due to the presence of the L-amino acid oxidase in soluble form. When high biotin extracts are dialyzed or precipitated with ammonium sulfate, L-oxidase activity is observed, but there is only 0.1 to 0.25 as much as in a low biotin extract (Table VIII). The increased activity due to growth on low biotin is also demonstrated by the occurrence of the enzyme in the culture medium under conditions of biotin limitation only (1).

Biotin has been found to have a particularly close relationship with the formation of the L-amino acid oxidase. The effect of the reduction of biotin level in the medium is not reproduced consistently by any other modification of cultural conditions yet tried. The relationship of biotin to formation of the L-oxidase in minimal medium will be considered in the subsequent

Table VIII

L-oxidase Activity of High and Low Biotin Extracts

Crude extracts--strain 4A, six day cultures. Dialyzed and precipitated extracts--strain 25R10, seven day cultures. Substrate, M/270 L-leucine. Theoretical oxygen uptake--93 µl. t=time after mixing.

Type of Extract	t (Mins.)	Total oxygen uptake (µl.)					
		High biotin			Low biotin		
		Endog.	+Leu-cine	Δ	Endog.	+Leu-cine	Δ
Crude	10	-	-	-	12	15	3
	60	12	15	3	60	103	43
	120	26	30	4	83	171	88
	170	33	39	6	100	196	96
Ppted.	15	-	-	-	0	12	12
	45	1	8	7	4	47	43
	85	3	21	18	10	98	88
	135	7	37	30	15	111	96
Dialyzed	10	1	3	2	2	18	16
	35	9	20	11	10	89	79
	85	19	51	32	23	118	95
	115	27	76	49	33	129	96

parts of this section. Other important evidence will be presented in the discussion of the adaptive formation of the enzyme.

Several approaches to a study of the mechanism by which L-oxidase activity of mycelium is increased by the limitation of biotin have been considered. One of the first was the observation (1) that certain strains did not show a biotin effect. After exploration of this possibility, work was continued on possible biochemical relations between biotin and the enzyme.

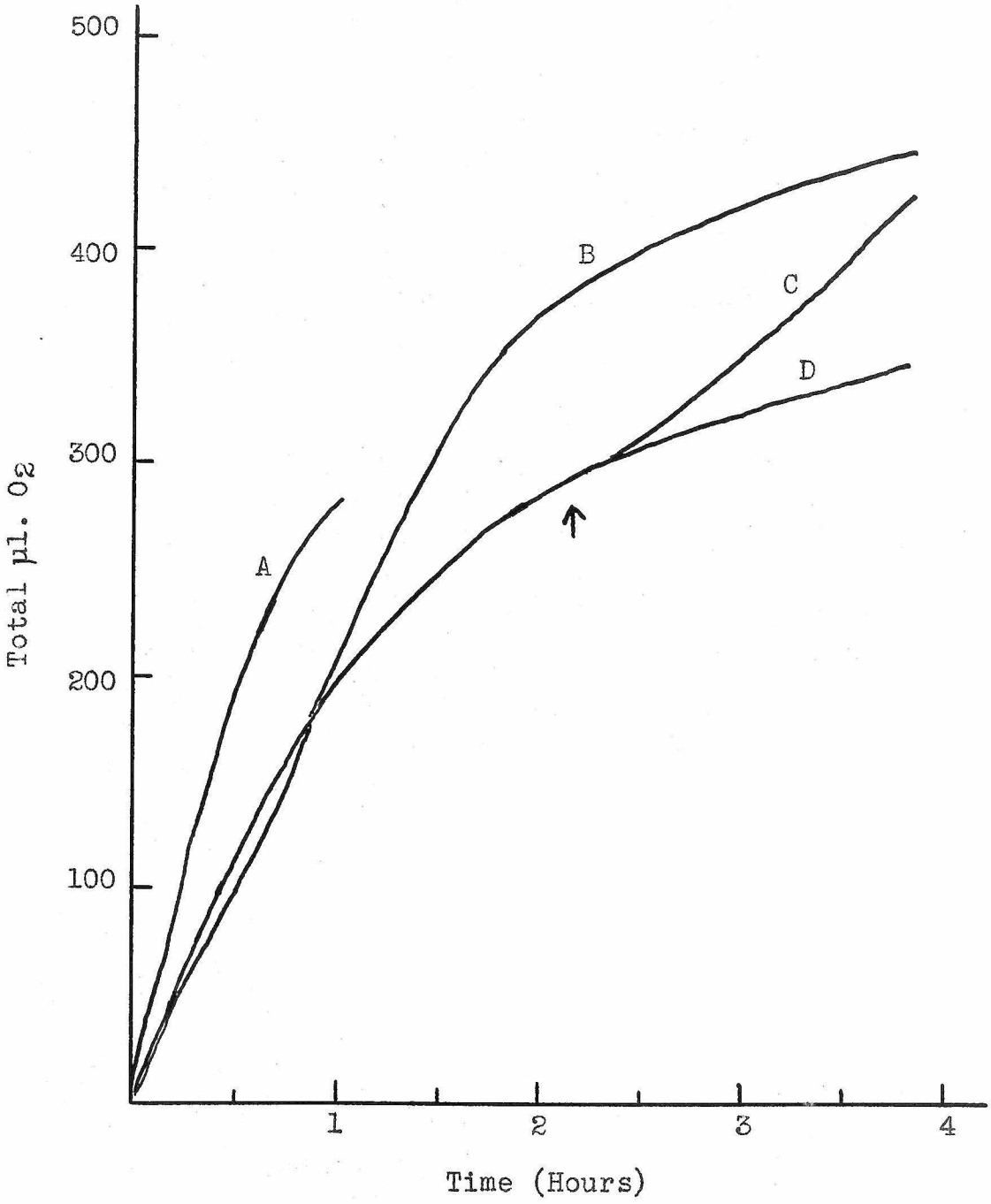
Strain Differences in Response to Low Biotin

The results of Bender et al. (1) indicated that under identical conditions some wild type strains of Neurospora produce only a D-amino acid oxidase (4), some only an L-amino acid oxidase, while others form both enzymes. Further investigation, described below, has shown that while quantitative differences probably exist among the strains with respect to production of the enzymes, there is no absolute difference among them, and both oxidases can be demonstrated in every strain tested.

Crude extracts of Neurospora cultured in a low biotin medium show a high background rate of oxidation as compared to extracts prepared from cultures grown in the standard medium (Cf. Table VIII). This oxidation may remain constant in rate for periods of time varying from a few minutes to several hours before it diminishes. If an L-amino acid is added during the constant period, no increase in the rate of oxidation is observed, but the decline in rate is postponed. If, however, the background rate is allowed to fall off somewhat before the

amino acid is added, then an immediate increase in oxygen uptake is obtained (Fig. 4). In either case, an extra quantity of oxygen is consumed equivalent to the substrate added. These results suggested that the background oxygen consumption of the extracts is due chiefly to the oxidation of endogenous L-amino acids which, at the outset, saturate the system so that further addition of L-amino acids is without effect on the oxidation rate. This conclusion is supported by the following evidence: 1. Neurospora extracts contain 8 to 35 micromoles of free amino acids per gram of fresh mycelium, as determined by the colorimetric ninhydrin method (30). Amino acids attacked by the L-oxidase constitute 40 to 80% of the total. The presence of alanine, methionine (or valine), the leucines (or phenylalanine), and others has been demonstrated by paper chromatography. (See also Fling and Horowitz (31).) 2. Extracts which have been purified by ammonium sulfate precipitation or dialysis show immediate oxidation of added L-amino acids with little or no background oxygen consumption (See Table VIII). 3. If, to a boiled low biotin extract, some highly active L-oxidase precipitated from the culture medium is added, a quantity of oxygen is consumed which approximates that consumed by the crude unboiled extract. 4. The addition of a D-amino acid to crude extracts during the constant phase of the endogenous respiration results in an immediate increase in the oxidation rate (Fig. 4).

Figure 4



Crude Low Biotin Extract; L- and D-oxidase Activity

Added: Curve A, 0.25 ml. M/30 D-methionine, at zero time; Curve B, 0.25 ml. M/30 L-phenylalanine, at zero time; Curve C, 0.25 ml. M/30 L-phenylalanine, at 132 minutes (arrow); Curve D, 0.25 ml. H_2O, at zero time. pH 8.

With the above facts in mind, a reinvestigation was made of strains which, on the basis of the earlier tests, had appeared to be devoid of the L-oxidase. In every case it was found that the enzyme is easily demonstrated after the background oxidation enters the diminishing phase. At the same time, it was found that the enzyme could be detected in the medium (low biotin) of all tested strains if the enzyme is concentrated by precipitation and resuspension in a smaller volume as described above.

Effect of Biotin on L-oxidase Activity in vitro

The possibility that biotin might be an inhibitor of L-oxidase activity, was first considered as an explanation of the biotin effect. Biotin was added to an aliquot of a low biotin extract, and its effect on endogenous respiration and oxidation of an added substrate determined. The background respiration, itself a measure of L-oxidase activity (See below), was slightly increased, as was the oxygen uptake with added phenylalanine (Table IX). It is apparent that biotin in not inhibitory to L-oxidase activity in vitro. Other tests for inhibitors of the enzyme will be considered later.

Extractability of the Enzyme

The effect of the lowered biotin content of medium might be to increase the solubility of the L-oxidase without affecting the total L-oxidase activity of the mycelium. It has been shown that there is an increased total production of L-oxidase on low biotin

Table IX

Effect of Biotin on L-oxidase Activity in Vitro

Crude extract of Sh-Abbot-a, grown 8 days in low biotin minimal. Biotin added (0.1γ/vessel) to extract before eqilibration. t=time after addition of substrate (0.25 ml. M/30 L-phenylalanine).

t (hrs.)	Total oxygen uptake (µl.)					
	Endogenous		+Phenylalanine		Excess	
	Control	+biotin	Control	+biotin	Control	+biotin
1	141	134	123	114	-18	-19
2	302	311	320	307	18	-4
3	361	380	440	448	79	68

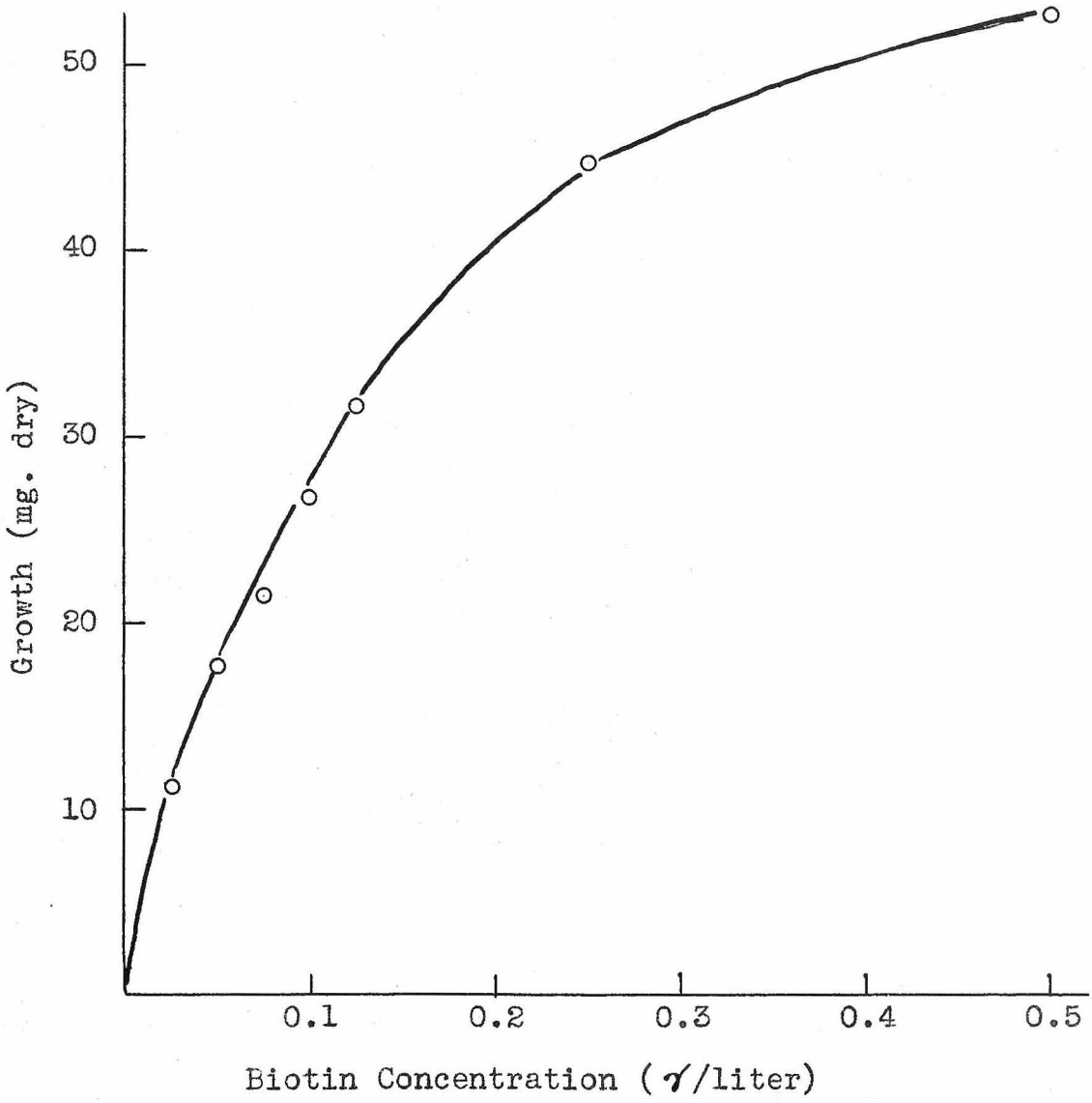
medium. If the greater L-oxidase activity of low biotin extracts were due to increased solubility of the enzyme, the residues remaining after extraction of high and low biotin pads would be expected to show a difference in L-oxidase activity opposite to that observed with corresponding extracts. Washed residues of high and low biotin mycelium were tested for L-oxidase activity. With L-phenylalanine as substrate, the high biotin residue catalyzed the uptake of oxygen at the rate of 0.1 µl./hr./mg. dry material, while the rate for the corresponding low biotin residue was 0.8 µl./hr./mg. (Rates are uncorrected for slight endogenous respiration.) Since the L-oxidase activity of the insoluble mycelial material is increased by lowered biotin by the same factor as is the activity of extracts, it is obvious that the

total activity of the mycelium is increased by a similar factor. The effects noted with extracts are not due to a change in the solubility of the enzyme.

Biotin and Growth

It was of interest to determine whether growth of the mold is actually limited by biotin in the low biotin medium. Growth of wild type strain 8a was therefore determined as a function of the biotin concentration, growth being measured after 72 hours at 25° (Fig. 5). It was found that the growth curve rises steeply as the biotin concentration increases, reaching a plateau in the neighborhood of 0.5γ biotin/liter. Beyond this point, further addition of biotin up to at least 2γ/liter had no effect on growth. At a concentration of 0.25γ/liter (the concentration of biotin in the "low biotin" medium) growth was 85 % of maximal. In experiments with other strains in which growth at a biotin concentration of 0.25γ/liter was compared with that at 5γ/liter, it was found that growth is frequently maximal at the lower concentration, even for growth periods as long as 2 weeks. It is concluded that 0.25γ biotin/liter is neither much in excess of the requirement for normal growth, nor is it seriously limiting. It can be described as marginal. This is in essential agreement with previous work on the biotin requirement of Neurospora by Ryan, Beadle, and Tatum (32) and by Hodson (33). The above results indicate that increased L-oxidase production cannot be ascribed

Figure 5



Biotin Response of Strain 8a

70 hours growth

to reduced growth. Further evidence has been obtained to support this view. If the biotin effect were wholly non-specific and merely the result of limited growth, it would be expected that L-oxidase production would be increased when growth is limited by other conditions than biotin level. A mutant, No. 85518, which is unable to reduce sulfate, but which grows readily when supplied with thiosulfate, was grown on super-optimal and limiting concentrations of thiosulfate (1.5 and 0.75 x 10^{-4}M) for seven days. Growth at the lower concentration was 60% of that produced with excess thiosulfate. No difference in background respiration was observed with crude extracts of these cultures, nor was there any measurable L-oxidase activity in either. Burton reports a loss of 72% in total L-oxidase activity in inositol-less strain CMI 17836 when grown on a concentration of inositol which limited growth to 60%. Activity was reduced 85% when growth of a wild type was limited to 80% by the omission of iron. It can thus be stated that limitation of growth, per se, is not sufficient to increase L-oxidase activity.

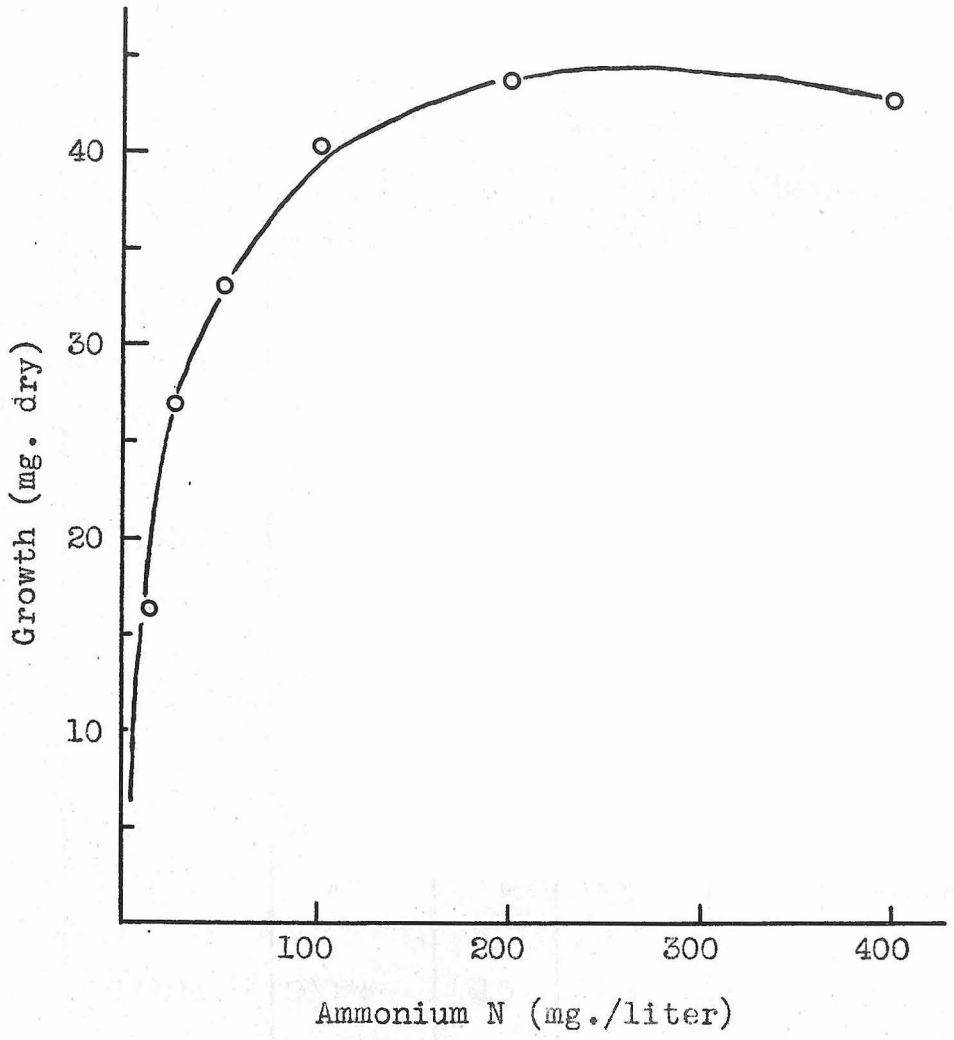
Nitrogen Limitation

The next possibility was suggested by the work of Winzler, Burk and du Vigneaud (34) which showed that biotin was required for the uptake of ammonia by yeast cells. If the same relationship existed in Neurospora, it might be presumed that nitrogen limitation would have the same effect as biotin limitation. That is, L-oxidase production may be increased when biotin is limited because

of an impairment of ammonia uptake. Therefore, a decrease of available ammonia in the presence of excess biotin might give the same result.

A limiting concentration of ammonia was determined from growth experiments (Fig. 6). About 50 mg. N/liter is the concentration at which the growth curve begins to level off. (This agrees with the figure given the author by B. S. Strauss and used as the basis for the earlier experiments.) Enzyme determinations have been done with cultures grown on nitrogen concentrations of 53, 105, 525, and 1110 mg. N/liter supplied as ammonium sulfate or chloride, and with biotin at 5 γ/liter. The first experiments with the lowest concentration seemed to show an increase in L-oxidase. There was measurable oxygen uptake due to the addition of substrate to crude extracts, which was never observed with similar preparations grown on minimal medium. More rigorously performed experiments have shown however that such a response is not typical (Table X), though other cases of apparent increase in L-oxidase activity due to nitrogen limitation have been observed. In none of the positive results is the magnitude of the effect comparable to that obtained by biotin limitation. In fact, the most nearly reproducible response to the limitation of nitrogen supply in these and other experiments is a decrease in L-oxidase activity. The data suggest, however, that other variables have an effect on the activity observed, one being the increase

Figure 6



Ammonium Response of Wild Type 4A

92 hours growth. Nitrogen-free minimal plus $(NH_4)_2SO_4$.

Table X

Effect of Ammonium Level on L-oxidase Activity in Extracts

	Nitrogen-free minimal with added $(NH_4)_2SO_4$.				
Exp't No.	Conc. of N mg./l.	Culture volume (ml.)	Age (days)	Growth (mg. dry)	Activity µl. O_2/hour/mg. DWE
1	1055	200*	7	315	0.09
	525	"	"	290	0.17
	105	"	"	275	0.07
	53	"	"	300	0.07
2	1110	400**	6	770	0.10
	525	"	"	755	0.10
	1110	200**	"	580	0.40
	525	"	"	540	0.15
3	1110	500**	15	725	0.99
	53	"	"	605	0.31
	1110	200**	"	295	1.13
	53	"	"	290	0.15
4	1110***	200*	10	235	1.28
	53***	"	"	255	0.41
	53	"	"	265	0.04

* In 750 ml. Erlenmeyer flask. Depth of medium, 2 cm.
** In Fernbach flask. Depths: 200 ml., 1 cm.; 400 ml., 1.8 cm.; 500 ml., 2 cm.
*** Low biotin; all others high biotin.

produced on shallow medium (Experiment 2). The high activities found in Experiments 3 and 4 seem to be due to the age of the culture, since it has been found in other experiments that appreciable amounts of L-oxidase are eventually formed on a high biotin medium. The final experiment shows a 3 fold reduction of activity by nitrogen limitation in low biotin culture. However, a 10 fold difference was found between high and low biotin cultures both grown on limiting nitrogen.

It is apparent that nitrogen limitation is not equivalent to biotin limitation in terms of the production of the L-amino acid oxidase.

Aspartic Acid

One other way in which biotin and nitrogen metabolism are known to be related is in the synthesis of aspartic acid.

In many bacteria, aspartic acid exerts a sparing action on the amount of biotin required for optimum growth. Other work has shown the need for biotin in fixing CO_2 into oxalacetic acid (Reviewed in (35)). Hodson (36) tested the ability of aspartic acid to replace biotin for Neurospora or to increase growth in the presence of biotin. No significant effect was found. The effect of aspartic acid on production of the L-oxidase in low biotin medium has been tested (Table XI). Amounts used exceed those needed for replacement of biotin for bacteria. No decrease of L-oxidase activity was produced, and there

Table XI

L-oxidase Activity of Mycelium Grown on Low Biotin Minimal plus Aspartic Acid

Supplement	Growth	Activity μl. O_2/hr./mg. DWE	Yield μl. O_2/hr./pad
None	315	0.31	98
L-aspartate 125 mg./l.	315	0.41	129
" 250 "	400	0.33	132
" 500 "	410	0.49	201

is a slight increase at higher levels of aspartic acid. A decrease would be expected if biotin limitation produced an aspartic acid deficiency which in turn led to an increase of the L-oxidase. The increase in growth at the higher levels may be significant, although many amino acids, when added to minimal medium, increase the growth of wild type strains.

Biotin and Riboflavin Metabolism

The effect of biotin on riboflavin metabolism was considered next on the hypothesis that biotin might function in a system which led to the destruction of riboflavin. Pads grown on high biotin might lack sufficient riboflavin for the production of the prosthetic group of the enzyme. The situation looked for would be analogous to that found by Bhagvat and Devi (37) in the rat, in which biotin deficiency increases the riboflavin content

of liver. Accordingly, the effect on L-oxidase production of large amounts (2-10 mg./liter) of riboflavin in high biotin medium was tested. Extracts of mycelia grown in such media showed no measurable L-oxidase, nor any significant increase in background respiration, a characteristic of low biotin extracts. Nor did the addition of riboflavin to high biotin extracts (10γ/2ml.) alter the oxygen uptake significantly. Addition of riboflavin to high biotin medium did not cause the production of L-oxidase in the medium after 21 days growth, nor did its addition to low biotin medium increase the amount of enzyme produced. Fluorometric measurement of riboflavin containing media, on which the mold had grown for various periods of time up to six days, indicated that there are no appreciable differences in riboflavin uptake on high and low biotin.

Biotin and D-oxidase Activity

Also considered in this connection has been the possibility of a relationship between the D-amino acid oxidase and the L-oxidase. Unfortunately, time has prevented a full exploration of this problem. In four different experiments the D-oxidase activity of pads grown on low biotin minimal medium was 38 to 77% of the activity found in corresponding pads grown on high biotin. The average activity was 55%, or a reduction of 45% due to limitation of biotin. If the initial endogenous respiration of these same extracts is taken as a measure of L-oxidase activity, the average L-oxidase activity in the

low biotin extracts was 8 times that of the high biotin extracts. The magnitude of the difference is probably even greater, since the estimates of oxidase activity were made with crude extracts. (It was necessary to use crude extracts, since it has been found that D-oxidase activity is lost completely when extracts are dialyzed, stored in the cold overnight, or precipitated with 0.8 saturated ammonium sulfate. The D-oxidase activity of a precipitated extract was not restored upon the addition of a boiled aliquot of the original crude extract or of a boiled L-oxidase preparation. Immersion for three minutes in boiling water, as done here, is sufficient to release FAD from the L-oxidase (9).)

An L-oxidase Inhibitor

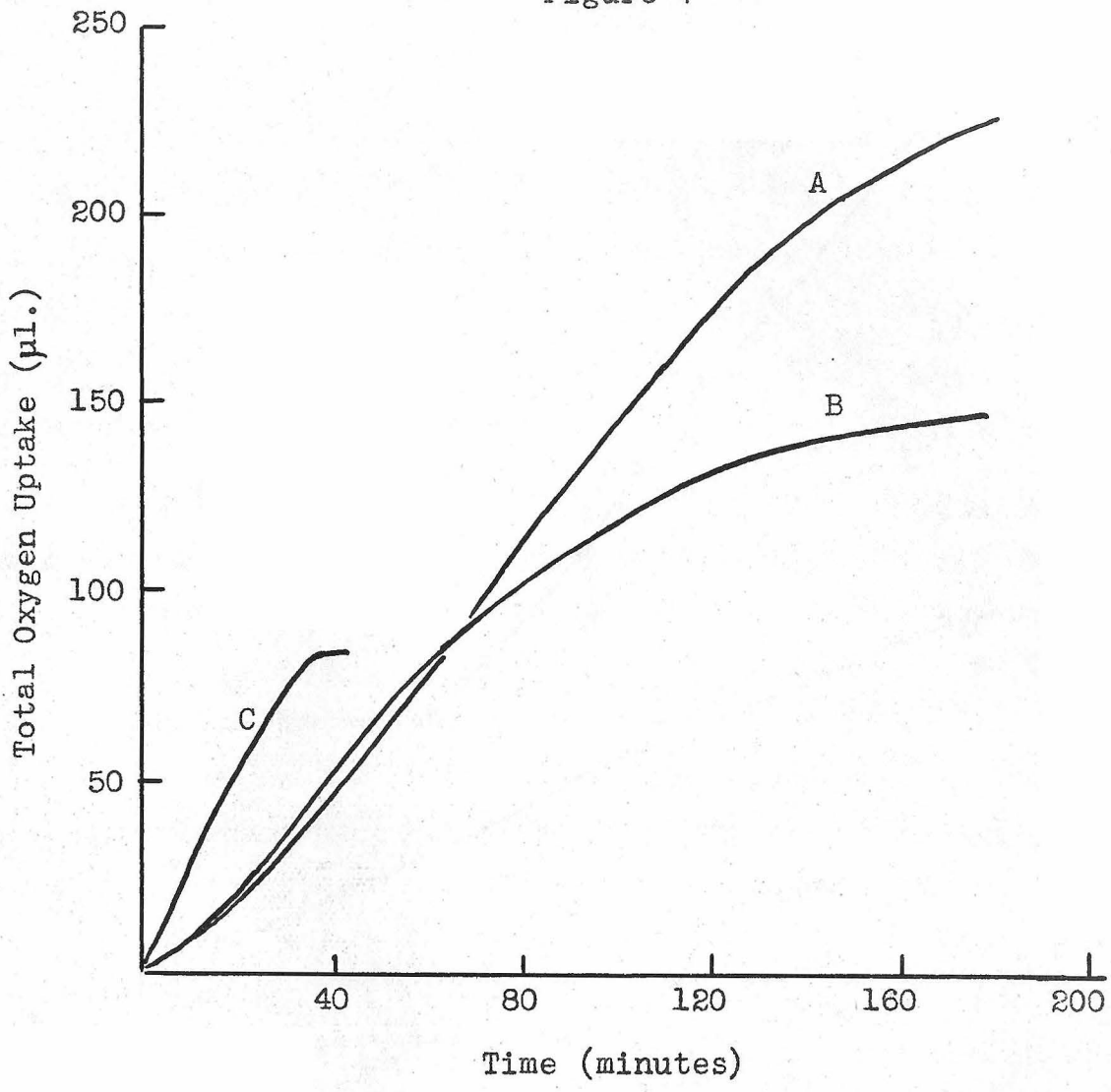
The possible formation of an inhibitor of the L-oxidase as a direct function of biotin concentration was considered in some detail. If similar amounts of enzyme are formed on high or low biotin, the low activity in extracts of high biotin cultures could be due to the presence of an inhibitor. Such an inhibitor has been tested for in several ways. As noted above, biotin has no inhibitory effect on L-oxidase activity *in vitro*.

After dialysis or ammonium sulfate precipitation a high biotin extract is still many times less active than a low biotin extract (See Table VIII). This eliminates the possibility of a low molecular weight dissociable inhibitor, which should be removed from the extract by

either procedure. The results of a test for a heat stable inhibitor are presented in Figure 7. In this experiment, lyophilized L-oxidase from the medium was added to crude, boiled, high biotin extract with and without the addition of leucine. The resulting curves of oxygen uptake are seen to be very similar to those obtained in a crude low biotin extract (Cf. Fig. 4). The "background", due to amino acids in the boiled extract, rises rapidly at first, then gradually falls off. The oxidation of "exogenous" substrate is not discernible until the "background" begins to decline. Without extract the oxidation of leucine by the same amount of lyophilized enzyme proceeds more rapidly, which can be attributed to the absence of competing substrates, as with dialyzed low biotin extracts. It is concluded that there is no heat-stable inhibitor of L-oxidase activity in high biotin extracts.

The results of experiments testing for the presence of a heat-labile inhibitor in a high biotin extract are shown in Figure 8. Two types of tests were made. In one, lyophilized L-oxidase was added to a crude high biotin extract. This resulted in an immediate increase in oxygen uptake. The increase was 2.1 fold in 1 hour and 2.7 fold after 3 3/4 hours. The rate was unchanged at the end of the run and another flask with leucine added showed no excess oxygen uptake. A final test for a heat-labile, non-dialyzable, inhibitor was performed with two dialyzed extracts from pads grown on casein hydrolysate

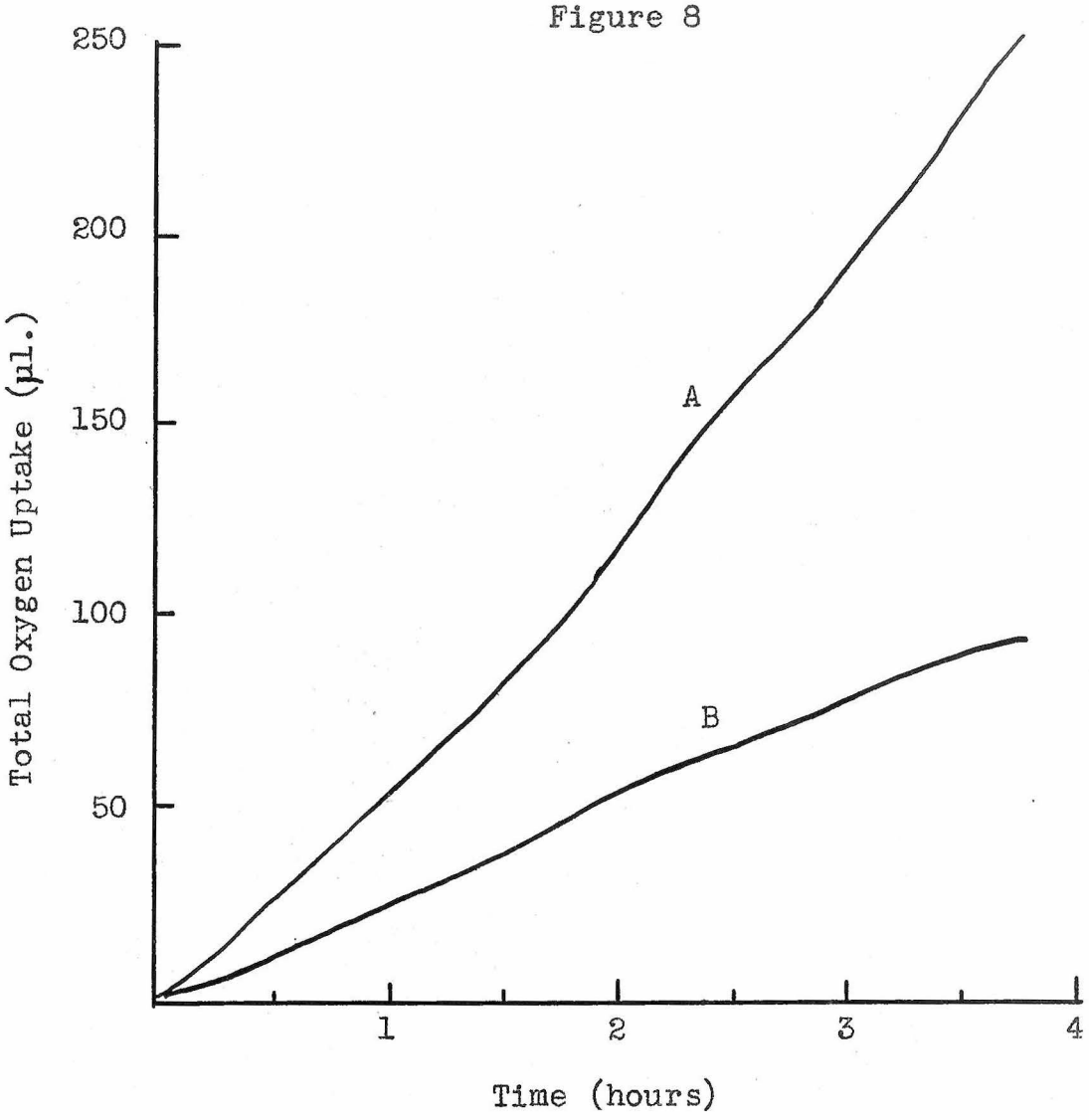
Figure 7



Test for Heat-stable Inhibitor

Curve A, 1 ml. boiled high biotin extract + 1 ml. buffer + L-oxidase + L-leucine; Curve B, 1 ml. boiled high biotin extract + 1 ml. buffer + L-oxidase + H_2O; Curve C, 2 ml. buffer + L-oxidase + L-leucine. (Lyophilized L-oxidase, 2 mg./vessel; L-leucine, M/300.) Extract of 6 day culture of 4A.

Figure 8



Test for Inhibitor in Crude Extract

Curve A, crude high biotin extract + 2 mg. lyophilized L-oxidase; Curve B, crude extract alone. No added substrate. Extract of 6 day culture of 4A.

as sole nitrogen source with biotin at 5.0 and 0.5 γ/ liter. The activity of the high biotin extract was 1/100 that of the low biotin extract. One ml. of the high biotin extract was added to one ml. of the low biotin extract one half-hour before the addition of the substrate. The rate of oxidation of the substrate (L-leucine) was not more than 15% lower for the mixed extracts than it was for the low biotin extract alone (Figure 9).

It is concluded that there is little evidence for an L-oxidase inhibitor in high biotin extracts. The possibility that a firmly bound inhibitor is present still exists.

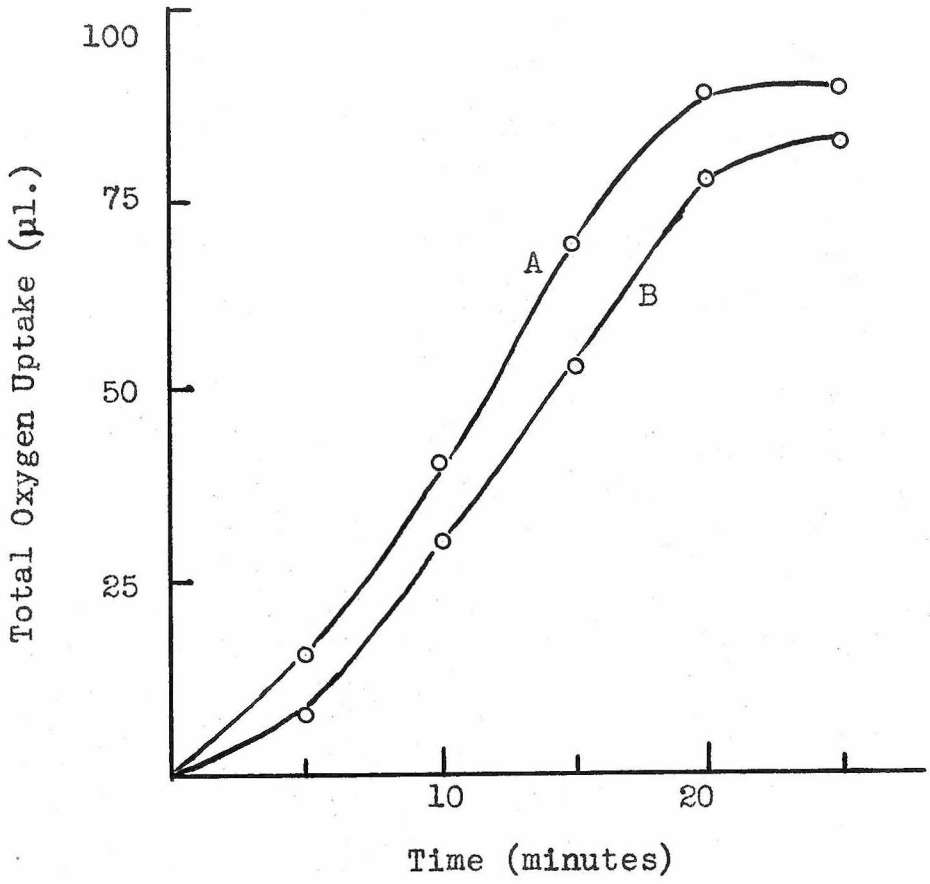
V. Adaptive Formation of the L-amino Acid Oxidase

It has been shown that the effect of biotin on production of the L-oxidase is not mediated through: (1) a general effect on growth; (2) lowered ammonia assimilation; (3) impaired aspartic acid or riboflavin metabolism; or (4) the formation of a readily detectable inhibitor of L-oxidase activity. As will now be shown, a clue to the mechanism of the biotin effect has been found in the adaptive formation of the enzyme, and in the relation of biotin to this phenomenon.

Preliminary Observations

When wild type Neurospora is grown on hydrolyzed casein as a sole nitrogen source, the L-oxidase activity measured in an extract of the culture is greatly increased

Figure 9



Test for Non-dialyzable Inhibitor

Substrate, M/270 L-leucine; pH 8. Curve A, 1 ml. active extract + 1 ml. buffer; Curve B, 1 ml. active extract + 1 ml. inactive extract. (See text for details.) Strain 4A.

over that of a corresponding culture grown on minimal medium (Table XII). The effect is noted when the two cultures used have been grown on high or on low biotin. When a culture is grown on low biotin and casein hydrolysate, the effects of low biotin and of casein on production of the enzyme are synergistic.

Table XII

Adaptive Formation of the L-amino Acid Oxidase

Strain 4A, grown six days on 200 ml. medium in Fernbach flasks. Dialyzed extracts. Minimal medium and nitrogen-free minimal medium plus 1% casein hydrolysate. Substrate, L-leucine.

Medium	Biotin γ/l.	Growth mg. dry	L-oxidase activity		L-oxidase yield/pad
			µl. O_2/hour/mg. DWE	Relative	
Minimal	5.0	1710	0.02	1.0	34
"	0.25	945	0.17	8.5	161
Cas. hydr.	5.0	1430	0.19	9.5	272
"	0.25	1250	2.28	114.0	2845

Criteria of Enzymatic Adaptation

The increase of L-oxidase activity produced by the presence of exogenous amino acids appears to be the same type of effect observed in adaptive enzyme formation in other micro-organisms. Just as with yeast or bacteria, however, the same result could be produced by the selection, during adaptation, of a mutant with inherently high L-oxidase activity. To discriminate between the latter

possibility (cultural adaptation) and enzymatic adaptation (an actual increase of enzyme content in cells of unchanged genotype), various experiments can be performed (38, 39). One, the Luria-Delbruck variance analysis (40), is not readily applicable to Neurospora because of the mold's habit of growth. In many cases, an adequate demonstration of enzymatic adaptation can be made by showing increased formation of the enzyme in the total or virtual absence of growth of the adapting culture. The opposite effect, loss of the enzyme from an adapted culture upon removal of the adaptive substrate, can be taken as evidence for enzymatic adaptation if proper precautions are taken. If growth occurs during deadaptation, it must be shown that the loss of activity is not merely due to dilution of the enzyme: if the adapted culture consisted of highly active mutant cells which had been selected for during adaptation, deadaptation might consist of the selection of wild type cells of low activity. Growth of these wild type cells would dilute the enzyme, producing a decrease of activity per unit weight of culture.

In addition to these microbiological criteria, certain enzymological conditions must be fulfilled before a specific case can be termed enzymatic adaptation. It must be demonstrated that an actual increase of enzymatically active protein has been produced during adaptation. This can best be demonstrated by the use of cell-free preparations in determining enzyme activity. In addition, the adaptive

process must be shown to be substrate-specific, that is, elicited only by compounds which are substrates of the enzyme involved.

The experimental results presented below meet enough of these criteria to show that the L-amino acid oxidase may be regarded as an adaptive enzyme. In addition, the effects of certain cultural factors on the adaptive response are described.

Deadaptation

The most clearcut evidence that the increased L-oxidase activity of mycelium grown on amino acids represents enzymatic adaptation is given by deadaptation experiments. To obtain as high an initial activity as possible, cultures to be tested were adapted on low biotin-casein hydrolysate medium (See Table XII). Deadaptation has been induced in this type of culture in two ways: (1) by the usual method of deadaptation, removal of the adaptive substrate; and (2) by the addition of an excess of biotin to an adapted culture (Table XIII). There is significant deadaptation in both cases, as shown by the 72 and 55% reductions of specific activity (Experiment 1). There is also a great net decrease in the total activity of a deadapted culture.

The decrease in total activity is conclusive evidence for the absence of selection. This effect could not be produced by selection and growth of an enzyme-free wild type, and dilution of the enzyme already formed.

Table XIII

Deadaptation by Removal of Amino Acids

and by Addition of Biotin

Cultures of 4A grown on 200 ml. low biotin nitrogen-free minimal plus 1% casein hydrolysate. Biotin addition: 1 γ biotin added to culture. Nitrogen source change: medium decanted and replaced with 200 ml. low biotin minimal. Assayed after six days total growth, extracts dialyzed. Substrate, L-leucine.

Exp't No.	Change	Culture age at change of medium	Growth mg. dry	Activity $\mu l. O_2$/hour/mg. DWE	Yield/ pad
1	None*	-	365	2.38	865
	None	-	385	2.32	895
	Biotin	4	500	0.66	330
	Minimal	4	475	1.06	510
2	None	-	605	1.31	795
	Biotin	5	620	0.74	455
	"	4	660	0.42	280

* Assayed after four days.

Even if the deadaptation consisted of the complete cessation of growth of a mutant of high activity and selection and subsequent growth of a wild type with zero activity, the total activity of a deadapted culture should still be 865 ul. O_2/hour/pad. The specific activities of the deadapted cultures would then be 1.73 and 1.82 respectively, instead of the observed values of 0.66 and 1.06. Experiment 2, and others not reported, confirm the deadaptive effect of excess biotin.

Deadaptation is seen to be explained by the loss of enzyme already formed. It cannot be due simply to the action of selective forces on genetically different forms of high and low L-oxidase activity. As an extension of this conclusion, it is apparent that adapted cultures do not consist of mutant cells of inherently high L-oxidase activity.

Short-term Adaptation without Growth

A more direct means of ruling out the possibility of selection of a mutant during the process of adaptation is the demonstration of adaptation in the absence of growth. This has been done in the present case.

Three cultures of wild type 4A were grown on 200 ml. of low biotin minimal medium. After four days one culture was assayed for L-oxidase activity, while the medium was changed in the other two. The old medium of these cultures was decanted off and fresh, sterile medium (biotin-free minimal, 1% sucrose) added. The fresh medium for one culture was supplemented with 0.125% L-lysine hydrochloride. Enzyme activity of the two cultures was assayed after 22 hours further incubation at 25°. The results are shown in Table XIV. It is seen that the omission of biotin effectively prevents further growth. The 50% increase of L-oxidase activity in the lysine-supplemented culture, double that produced in the absence of added adaptive substrate, represents a

significant adaptive response. The differences in dry weight are not significant. The increase of L-oxidase activity in the culture without added lysine is probably attributable to the absence of biotin. (See later section on relation of biotin to adaptation.)

It is apparent that an adaptive increase of the L-oxidase can occur in the virtual absence of growth. This evidence, combined with that on deadaptation, indicates that the mechanism of this increase is enzymatic adaptation and not the selection of a mutant of high L-oxidase activity.

Table XIV

Adaptation in the Absence of Growth

No. 1, zero time control; No. 2, experimental control; No. 3, experimental (lysine added). 22 hours incubation after change of medium. (For details, see text.) All extracts dialyzed. Assay substrate, L-leucine.

Culture No.	Growth mg.dry	L-oxidase Activity		Yield/pad	
		$\mu l.O_2$/hour/mg. DWE	Relative	$\mu l.$/hour	Relative
1	198	1.44	100	286	100
2	207	1.85	128	384	134
3	209	2.20	153	460	161

Specificity of Adaptation

The adaptive response of the L-oxidase to casein hydrolysate is not due to any particular one of the amino acids present nor merely to the addition of biologically available amino nitrogen. This is indicated by experiments in which individual amino acids were added to low biotin minimal medium, and compared with the effect of the addition of casein hydrolysate. Amino acids tested include leucine, lysine, histidine and glutamic acid (Table XV), and aspartic acid (See Table XI).

Concentrations of the amino acids used in the experiments of Table XV were chosen to give, respectively: (1) the same amount of the amino acid as does 0.1% casein (41); and (2) α-amino nitrogen equivalent to the total nitrogen content of 0.1% casein. Examination of the data shows four points of interest: (1) little or no increase of the enzyme is produced at the lower concentration of any of the amino acids; (2) a significant increase is elicited only by those which are good substrates for the enzyme; (3) the magnitude of such an increase varies with the amino acid used; and (4) histidine, although a good substrate for the enzyme in vitro, induces little adaptation. Aspartic acid was shown in Table XI to produce a slight increase of L-oxidase activity. One may conclude: (1) that the adaptive formation of the L-oxidase is dependent upon the presence of an attackable substrate in sufficient concentration; (2) that the effect of casein

Table XV

Specificity of Adaptive Response of L-oxidase

Cultures of 4R10 grown 6 days in 200 ml. low biotin minimal medium. Precipitated extracts.

Exp't No.	Supplement	Activity µl. O_2/hour/g. FWE	Yield/ pad
1	None	56	98
	0.1% casein hydrolysate	152	228
	0.025% L-glutamic acid	45	86
2	None	47	66
	0.1% casein hydrolysate	89	124
	0.15% L-glutamic acid	49	93
	0.01% L-leucine	30	45
	0.15% L-leucine	137	233
3	0.004% L-histidine·HCl·H_2O	56	126
	0.225% "	76	171
	0.0095% L-lysine·HCl	76	152
	0.195% "	197	345
4	None	34	43
	0.15% L-leucine	188	235
	0.195% L-lysine·HCl	330	495

hydrolysate is not due to the presence of a mixture of amino acids *per se*, but to its content of substrate amino acids (glutamic acid, a poor substrate for the enzyme, is the major amino acid component of casein); and (3) that the availability of amino nitrogen is not a sufficient condition for adaptation. The latter point is borne out by the findings of Hungate (42) and Fling (43). They found that as sole nitrogen sources for wild type *Neurospora*, glutamic and aspartic acids were of relatively high activity, leucine gave about half as much growth, and lysine and histidine only slight growth.

Biotin and the Adaptive Response

The substrate-specificity of the adaptive formation of the L-oxidase has been demonstrated. However, the increased activity of the L-oxidase on low biotin minimal medium is, nominally, a non-specific effect. That it may not be wholly non-specific can be inferred from the results of experiments on the relation of biotin to the adaptive response.

As suggested earlier (Table XII), the concentration of biotin is an important factor in the magnitude of the L-oxidase activity produced by adaptation. This is more clearly shown by experiments in which several concentrations of biotin were used in medium containing casein hydrolysate as the sole nitrogen source. As shown in Table XVI, there is a 125 fold difference in the L-oxidase activity of cultures grown, respectively, on

Table XVI

Effect of Biotin Concentration on Adaptive Response to Amino Acids

All cultures grown on 1% casein hydrolysate, 6 days. Extracts dialyzed. Strain 4A.

Biotin conc. γ/l.	Growth mg. dry	Activity µl.O_2/hr./mg. DWE	/g. FWE	Yield µl.O_2/hr./pad
5.0	640	0.04	6	27
1.0	615	0.98	155	602
0.5	650	3.84	695	2495
0.25	425	5.10	850	2165

0.25 and 5.0γ of biotin per liter. Intermediate activities are produced at intermediate concentrations of biotin.

Despite the low activity observed above with the highest concentration of biotin, an increase of the enzyme is usually observed in high biotin (5γ/liter) cultures grown on casein hydrolysate as compared to inorganic nitrogen. In eight experiments, performed with two wild type strains grown on different volumes and depths of medium, and with different periods of incubation, such an effect has been observed six times (Table XVII, see also Table XII). In one case, there was a decrease of activity with the use of amino acids as a nitrogen source; and in the other, no significant difference in L-oxidase activity was observed. In the six positive cases, the increase

Table XVII

Adaptive Response on High Biotin Medium

All casein hydrolysate cultures grown on nitrogen-free minimal supplemented as indicated. Control cultures on minimal medium. Biotin--5.0 γ/liter. Strains 4A and 4R10. Substrate, L-leucine.

Exp't No.	Strain	Age in days	Cas. hydr. conc.(%)	Culture Volume (ml.)	L-oxidase activity $\mu l.O_2$/hour/g. FWE
1	4R10	8	0	200*	19
	"	"	0.75	"	58
2	4A	7	0	500**	23
	"	6	0.75	"	13
3	4A	7	0	200*	19
	"	"	0.75	"	16
	4R10	7	0	"	17
	"	"	0.75	"	27
4	4A	7	0	200*	7
	"	"	0.36	"	63
5	4A	14	0	400**	178
	"	"	0.75	"	717
	"	"	0	200**	92
	"	"	0.75	"	220

* In 750 ml. Erlenmeyer flask.
** In Fernbach flask. See footnote to Table X.

in activity ranged from 1.6 to 9.0 times with a mean of 4.7. The data suggest that conditions favoring an adaptive response on high biotin include shallow medium, and some optimum concentration of amino acids. In two of the positive cases, the period of incubation was 14 days as compared to 6 to 8 days for the others. The activities observed after longer incubation were comparable to those obtained with corresponding low biotin media. Two other experiments in which amino acids were added to high biotin minimal medium showed no increase of L-oxidase activity. Adaptive formation of the L-oxidase apparently does occur in high biotin medium, but only when other cultural conditions are favorable.

It is concluded that the degree of adaptive formation of the L-oxidase is controlled by biotin concentration. This is evident from the demonstration that excess biotin produces deadaptation comparable to that obtained by removal of substrate, as well as from the more direct tests.

As was pointed out previously, Neurospora mycelium grown on minimal medium contains free amino acids. A rather large percentage of these amino acids is attackable by the L-oxidase. (See also Table XXIII.) As a consequence of this fact and of the control of adaptation by biotin, it may be inferred that the increased L-oxidase activity observed on low biotin minimal medium represents an adaptation to intracellular free amino acids. Exogenous amino acids, added to produce demonstrable adaptation,

would thus be merely augmenting the stimulus already provided by endogenous amino acids.

Some indirect evidence may be adduced in support of this hypothesis. The decreased L-oxidase activity of mycelium grown on limited nitrogen (Table X), might be attributed to a decreased synthesis of amino acids, and a consequent decrease in free amino acids. Also, as will be noted later, strain CMI 3411, which has a very high L-oxidase activity on low biotin minimal, also has a significantly greater concentration of free amino acids than does strain 4A. Further work on these phenomena might provide adequate tests of the hypothesis of endogenously induced adaptive formation of the L-oxidase.

Adaptation and Growth

Two types of experiments have been done on the degree of L-oxidase adaptation induced by different concentrations of casein hydrolysate. In one, casein hydrolysate served as the sole nitrogen source, and in the other, ammonium chloride was added to give a constant total nitrogen concentration. Detailed work correlating substrate concentration and enzymatic response should be done with single amino acids. Casein hydrolysate is inhibitory to growth at high concentrations, and its varied components make specific analysis difficult.

When casein hydrolysate is used in low biotin medium as a growth-limiting nitrogen source, a low L-oxidase activity is observed (Table XVIII). Growth is

Table XVIII

Casein Hydrolysate as Nitrogen Source

and Adaptive Substrate

Cultures grown 6 days on 200 ml. low biotin, nitrogen-free minimal medium, supplemented with casein hydrolysate as indicated. Strain 4A. Substrate, L-leucine.

Cas. hydr. conc. %	Approx. mg.N/l.	Growth mg.	Activity	
			$\mu l.O_2$/hour/mg. DWE	$\mu l.O_2$/hour/g. FWE
0.1	150	355	0.36	76
0.25	375	445	1.83	354
0.5	750	520	1.62	402
1.0	1500	475	2.34	503

increased not more than 50% by higher concentrations, while L-oxidase activity increases by 350-550%. The highest activity is observed at a concentration which is even slightly inhibitory to growth. This can be interpreted as meaning that, at low concentrations, most of the amino acids are incorporated unchanged or their nitrogen utilized by other mechanisms than the L-oxidase (transamination, for example). A relatively small amount would be left free to stimulate L-oxidase formation. At higher concentrations, the more readily usable amino acids provide sufficient nitrogen for growth, and a larger proportion of free amino acids results.

A similar L-oxidase activity is produced when the intermediate concentrations of casein hydrolysate are supplemented with ammonium chloride (Table XIX). Comparison of these results with those of Table XVIII shows that the presence or absence of inorganic nitrogen has no significant effect on L-oxidase production at the two comparable concentrations of casein hydrolysate. This lack of effect was further demonstrated in an experiment in which varied amounts of ammonium chloride were added to 1% casein hydrolysate medium (Table XX). The highest concentration of ammonium chloride provided a 53% increase in the total nitrogen content of the medium. The results show a slight inhibition of growth at the higher concentrations of the added salt, with no marked effect on activity or yield of the L-oxidase.

Table XIX

Adaptive Response with Casein Hydrolysate as Partial or Complete Nitrogen Source

Cultures of 4A grown 5 days, in 200 ml. medium. Biotin, 0.25 γ/liter. Total nitrogen concentration, 1100 mg. N/liter. Extracts precipitated and dialyzed. Substrate, L-leucine.

Concentration (%) NH_4Cl	Cas. Hydr.	Growth g. wet.	L-oxidase activity µl. O_2/hour/g. FWE
0.42	0	1.15	45
0.28	0.24	2.30	333
0.14	0.48	2.40	420
0	0.72	1.80	635

Table XX

Effect of NH_4Cl on Adaptive Response

All cultures grown 6 days on 200 ml. low biotin, nitrogen-free medium plus 1% casein hydrolysate (ca. 1500 mg. N/l.), supplemented with NH_4Cl as indicated. Strain 4A. Substrate, L-leucine.

NH_4Cl (mg.)	mg. NH_4 N/l.	Growth mg. dry	Activity $\mu l.O_2$/hour/mg. DWE	Yield/ pad
0	0	405	5.12	2080
153	200	425	6.11	2600
306	400	395	6.16	2440
612	800	365	5.50	2000

Effect of pH of Medium on Adaptation

That there is an optimum pH range for adaptive formation of the L-oxidase is shown by the data of Table XXI. Maximum activity of the enzyme was observed at pH 5, while the greatest growth (wet weight) was at pH 6.

Table XXI

Effect of pH on Adaptive Response

Cultures grown 6 days on low biotin, casein hydrolysate (1%) sole nitrogen source. pH values adjusted before autoclaving. Strain 4A. Substrate, L-leucine.

pH	Growth g. wet	Activity $\mu l. O_2$/hour/g. FWE	Yield/ pad
4.1	1.70	407	690
5.0	2.00	650	1300
6.0	2.40	565	1350
7.0	2.00	480	960

Strain Differences

Considerable difference exists in the amounts of L-oxidase formed under adaptive conditions by different wild type strains (Table XXII). The L-oxidase activity of 19 strains (re-isolated from a cross of 4A x 25R11) was determined after 6 days growth on low biotin, casein hydrolysate medium. Six strains show a degree of adaptation different from the mean by more than one standard deviation. Further study would be required to determine the mechanisms producing such differences.

Strain CMI 3411

Burton has reported (9) that a wild type strain of Neurospora, CMI 3411, shows no increase in L-oxidase activity when grown on low biotin casein hydrolysate medium as compared to low biotin minimal medium. The strain does show a biotin effect, the activity on low biotin minimal being approximately 10 times that observed on high biotin minimal. It was of interest to determine whether the absence of a casein hydrolysate effect represented an inherent difference in the ability to adapt. Experiments done by the present author have suggested that this is not the case.

Burton's results were confirmed with crude extracts of 6 day old cultures. The activity on leucine of a culture grown on low biotin minimal was 4.52 µl. O_2/hour/mg. DWE; growth was 285 mg. dry weight; and total enzyme yield 1285 µl. O_2/hour/pad. A corresponding culture grown with

Table XXII

Strain Differences in Degree of Adaptation

All cultures grown 6 days on low biotin, nitrogen-free minimal plus 1% casein hydrolysate. Extracts dialyzed. Substrate, L-leucine.

Strain No.	L-oxidase activity µl. O_2/hour/g. FWE	Strain No.	L-oxidase activity µl. O_2/hour/g. FWE
11R1	570	11R11	320
2	610	12	520
3	335	13	625*
4	540	14	720*
5	290*	15	460
6	420	16	355
7	335	17	495
8	510	18	135*
9	385	19	700*
10	260*		
		Mean	450
		S. D.	150

* Deviation from mean more than 1 S. D.

casein hydrolysate had an activity of 3.54 µl. O_2/hour/mg. DWE; growth, 335 mg.; and total activity 1200 µl. O_2/hour/pad. The reduction in specific activity due to the use of casein hydrolysate is 22%, although the total yield is not significantly changed.

It is seen that the activity of the British strain on low biotin minimal is comparable to the higher activities obtained by adaptation of strains 4A or 4R10 on low biotin-casein medium. A very rapid uptake of a large amount of oxygen was observed during equilibration of the extracts of CMI 3411 in the Warburg vessels. The latter fact and the high enzymatic activity suggested that strain CMI 3411 might possess a much higher concentration of intracellular free amino acids. If maximal adaptation (to the intracellular amino acids) occurred on low biotin medium, the addition of further amino acids would have no effect. As an extension of the idea proposed previously, that the L-oxidase increase on low biotin is due to adaptation to endogenous amino acids, it is thus proposed that the amount so formed is, within limits, a function of the concentration of such amino acids.

Strain CMI 3411 was shown to be significantly different from 4A in its content of free amino acids oxidizable by the L-oxidase (Table XXIII). Seven day old cultures grown on high and low biotin minimal medium were extracted with 70% alcohol (5 volumes/g. wet weight). Each extract was centrifuged, and the supernatant evaporated to dryness at 60° <u>in vacuo</u>. The residue was redissolved in water to give the equivalent of 50 mg. dry weight per ml., and the amino acid content of 1 ml. aliquots determined with lyophilized L-oxidase. The concentration of oxidizable amino acids in high biotin mycelium of CMI 3411 is 1½ times as

Table XXIII

Free Amino Acids in CMI 3411 and 4A

Pads grown seven days, extracted with 5 ml. 70% alcohol/ g. fresh weight. Extract evaporated to dryness, redissolved in H_2O to give 50 mg. DWE/ml. 1 ml. aliquots oxidized with lyophilized L-oxidase, plus catalase.

Strain	Biotin	Growth mg.dry	Total $\mu l.O_2$	μM amino acids	μM/mg. DWE	μM/ pad
3411	High	270	85.7	7.65	0.153	41.0
	Low	235	98.1	8.76	0.175	41.0
4A	High	745	55.9	4.98	0.099	74.5
	Low	425	21.2	1.89	0.038	16.1

great as that of 4A, and in low biotin mycelium is nearly 5 times as great. Whether these alone are sufficient differences to produce the great difference in enzymatic activity, and the lack of effect of exogenous amino acids with CMI 3411 cannot be said.

CMI 3411 is an abnormal strain in several other respects. The abnormalities evident from Burton's and the present work include the following: higher biotin requirement for half-maximum and maximum growth, lower maximum growth, inability to conidiate, and difficulty of transfer on solid medium. A further point noted by the present author is the very low dry weight/wet weight ratios of pads of CMI 3411. The mean ratio for 4 pads used in the present study was 0.08 as compared with the "normal" value of 0.22 cited in Section II. Whatever the basic defects

of this strain may be, it is likely that the higher L-oxidase activity is due in part to a higher concentration of intracellular free amino acids.

Discussion

The control of adaptive formation of the Neurospora L-amino acid oxidase by biotin has been seen to offer a significant lead to a partial understanding of the "biotin effect", i. e., the increase of L-oxidase observed when cultures are grown on minimal medium containing a marginal amount of biotin. It has been suggested that the increased L-oxidase activity of low biotin cultures is due to enzymatic adaptation elicited by intracellular amino acids. In terms of induced or spontaneous adaptation, the action of lowered biotin is to allow a net increase of the enzyme, and that of increased biotin is to prevent it. The results of the deadaptation experiments imply a biotin-mediated destruction of activity. The addition of excess biotin to an adapted culture, without the removal of the adaptive substrate, causes a great loss of specific and total L-oxidase activity. This suggests that biotin may control an equilibrium between the active enzyme and an inactive form. The nature of the latter can be postulated as any one of a number of things, for example: another enzyme, an L-oxidase-inhibitor complex, or an inactive precursor. One tempting idea is the possibility of a relationship between the L-oxidase and the D-oxidase. The activity of the latter has been shown to decrease under conditions

which produce an increase in L-oxidase activity.

Another case of increased enzyme activity in nutritionally deficient Neurospora cultures is found in the work of Shen (44), and Horowitz (45) on tyrosinase. Wild type grown on limiting concentrations of sulfate has a much higher tyrosinase activity than when grown in minimal medium (which contains excess sulfate). The increased activity is at least partially due to the absence of a tyrosinase inhibitor which is formed by cultures grown on excess sulfate.

A situation similar to the L-oxidase-biotin relationship has been found by Nason, et al., in the effect of a deficiency of zinc or other metal ions on synthesis of certain enzymes in Neurospora (46, 47). Zinc-deficient mycelium is found to have a greatly increased diphosphopyridine-nucleotidase activity, while the concentration of certain other enzymes decreases, or changes only slightly. Similar effects are found in biotin or nitrogen deficiency. These authors propose the working hypothesis that:

> "those enzymes which increase in nutritionally deficient cells are 'primitive' proteins of relatively simple structure, the synthesis of which can proceed even in the absence of certain key reactions which are necessary for the building of more complex protein molecules." (46)

It is not clear just what is meant by "primitive" proteins, nor how one would test the hypothesis. More specific extensions of this hypothesis, as suggested above, would seem to be justified in the case of the Neurospora L-oxidase, in

view of the relationship of biotin to adaptive formation of the enzyme.

The finding of Blanchard, et al. (48), that biotin is required for the adaptive formation of the "malic enzyme" by Lactobacillus arabinosus is also pertinent to the present discussion. Biotin is apparently not a component of the functional enzyme, but would seem to function catalytically in its formation. Here is an analogy, albeit in reverse, with the apparent control by biotin of L-amino acid oxidase synthesis in Neurospora.

There is thus an implication from the work of Blanchard, et al., of Nason, et al., and of the present author that an important metabolic function of biotin may lie in its relationship to the synthesis of certain specific enzymes. This idea was presented by Blanchard, et al., as follows:

> "Only minute concentrations of biotin are required for bacterial growth. This substance is present in various tissues at very low concentrations relative to other vitamins which function as components of prosthetic groups of certain enzyme systems of intermediary metabolism (i. e. nicotinic acid, thiamine, pyridoxine, pantothenic acid). These facts make it unlikely that biotin is involved as a prosthetic group at the level of metabolism of the malic acid dissimulation system. It would appear that biotin functions at a more basic level and that it may be a component of the prosthetic group of an enzyme system which synthesizes (or is involved in the synthesis of) other prosthetic groups or apoenzymes." (48)

VI. The L-amino Acid Oxidase and Canavanine Resistance

Canavanine (NH_2-C(:NH)-NH-O-CH_2-CH_2-$CHNH_2$-COOH) (49, 50) is a structural analog of arginine, in which an oxygen atom replaces the terminal chain carbon of arginine. It is inhibitory to the growth of several microorganisms and higher plant tissues, and the inhibition is reversed be arginine. Organisms in which such a relationship has been demonstrated include certain wild type strains of Neurospora (2, 51); lactic acid bacteria and Escherichia coli (52); Avena (coleoptile growth and respiration) (53, 54); and maize (embryo growth) (55). Bacillus subtilis is resistant to canavanine (51). Other amino acids which reverse canavanine inhibition to some extent are: lysine (Neurospora, Avena, and maize); glutamic acid (Avena, maize); and ornithine and citrulline (lactic acid bacteria, maize). Canavanine inhibition of a partially sensitive strain of Neurospora is reversed by several other amino acids besides arginine and lysine, particularly methionine (2). A competitive relation between arginine and canavanine has been demonstrated in Neurospora and lactic acid bacteria (2, 52), and between lysine and canavanine in Neurospora (51).

Strain Differences in Sensitivity to Canavanine

The work of Horowitz and Srb (2) showed that there were marked differences in the canavanine sensitivity of different wild type strains of Neurospora. Growth of strain 25a was completely prevented by concentrations

of canavanine sulfate greater than 1.25 γ per ml. (5.55 x 10^{-6}M). A partially sensitive strain, 1A, was inhibited 55% by a ten fold greater concentration, and growth of resistant strain 4A was usually inhibited not more than 15% by concentrations up to 50γ per ml.

The difference in canavanine resistance between strains 25a and 4A was shown by these authors to be genetically determined. Nutritionally, however, the two strains are wild type, that is, capable of synthesizing all necessary amino acids, including arginine and lysine, from sugar and inorganic salts. Arginine and lysine are the only amino acids known to reverse canavanine inhibition of strain 25a, yet the ability to synthesize these compounds does not protect it from canavanine inhibition. This led Horowitz and Srb to suggest that:

> "the relative immunity to canavanine intoxication displayed by strain 4A results from a mechanism for detoxifying or otherwise disposing of the compound, a mechanism presumably possessed by strain 25a in a much less active form, if at all." (2)

That detoxification is involved in canavanine resistance was further suggested by the work of Teas (51, 56). He showed that canavanine is utilized by certain Neurospora mutants, apparently as a substitute for homoserine ($HOCH_2$-CH_2-$CHNH_2$-COOH), when the "cnr" gene, responsible for canavanine resistance, is present. He also showed that detoxification is probably not through the production of canaline (NH_2-O-CH_2-CH_2-$CHNH_2$-COOH), since canaline is inhibitory to many strains of Neurospora, regardless of

canavanine sensitivity (51). Canaline is formed from canavanine by the action of a liver enzyme, probably arginase (57). Preliminary experiments by the present author have shown that arginine and lysine are not significantly stimulatory to growth of canavanine sensitive strains, indicating that their sensitivity is not due to a decreased rate of synthesis of these compounds.

When it was found that canavanine is a highly active substrate for the L-oxidase, experiments were undertaken to determine if this enzyme could be part of the mechanism by which a resistant strain detoxified canavanine.

Strains used in the present work include: 4A and 4R10a, resistant; 25a, 25R8A, and 25R11a, sensitive; and 1A and 25R9A, partially sensitive; and in the final work, 22 cultures from random ascospores resulting from a cross of 4A and 25R11a. The re-isolated strains behaved similarly to the original strains with which they are classed here. It became necessary to use the re-isolated strains when the original culture of 25a became attenuated in growth and lost the ability to conidiate. (Other cultures of "25a" tried were not canavanine sensitive.)

"Keto-canavanine"

The L-oxidase produced in culture medium by either sensitive or resistant strains is very active against canavanine, oxidizing it to 80-95% of theoretical completion. The final solution after such oxidation shows a strong

color reaction for the presence of a keto-acid (13), as expected. A quantitative ammonia determination showed the production of 1 mole of ammonia per mole of substrate oxidized. It is suggested that the apparent lack of complete oxidation is due to the presence of desamino-canavanine, which can be spontaneously formed during the isolation of canavanine (58). It is also reported that desaminocanavanine is formed in solutions of canavanine which are allowed to stand for one to two weeks (16% conversion in 12 days at 28°). This may explain the low oxygen consumptions observed in the present work, since the solutions used were often stored (under refrigeration) for a few weeks. The desamino form would not be expected to react with the L-oxidase, if the cyclic structure proposed (58) is correct.

The product of the oxidation of canavanine by L-oxidase from strain 25a was tested for its effect on growth of 25a (Table XXIV). The preparations used were from two Warburg vessels in which oxygen uptake stopped at 90% of the theoretical. They were inactivated by immersion in boiling water, tested for the presence of keto-acid (positive), and sterilized by filtration. The results show that the oxidation product is not inhibitory to 25a, nor does the solution contain an antagonist of canavanine. Incomplete oxidation apparently does not leave a residual 10% of intact canavanine, since growth at the higher levels of "keto-canavanine" is not inhibited. This is further

Table XXIV

Growth of Strain 25a on Intact and Oxidized Canavanine

Supplement		Growth	
Canavanine sulfate γ/20 ml.	Oxid. products of canav. "γ"/20 ml.	Exp't 1 72 hrs.	Exp't 2 67 hrs.
0	0	67	38
25	0	±	0
0	25	67.5	36
0	50	73.5	35
0	100	64	35.5
0	250	69	30.5
0	500	-	33.5
25	250	-	0
50	250	±	0

evidence for the presence of an inactive compound in the canavanine used. Other experiments with re-isolated canavanine-sensitive strains have confirmed the non-toxicity of the oxidation products.

Since "keto-canavanine" is not inhibitory to strain 25a, it is clear that the L-oxidase could be the mechanism for detoxifying canavanine. However, the enzyme was found to be formed by all the strains involved, sensitive and resistant. Therefore, it was proposed as a working hypothesis that the sensitive strains might possess a modified L-oxidase which was incapable of acting on canavanine <u>in vivo</u>.

In vivo Activity of the L-oxidase

It has been difficult to devise an adequate test of the in vivo activity of the enzyme. Attempts were made to determine amounts of keto-acid produced by pads suspended in a relatively high concentration of canavanine. Keto-acid is formed, but the small amounts found indicate it is further broken down. Therefore, little reliance can be placed on the results.

Since the L-oxidase increases greatly on low biotin medium, resistance should be increased on low biotin medium, if the L-oxidase is the functional means of detoxification. That is, the concentration of canavanine required to produce a certain degree of inhibition would be higher in low than in high biotin medium. In testing this idea, canavanine was added to 2 day old cultures of strain 25R11 growing on high and low biotin. Total growth was determined after 2 more days of incubation. The results are expressed in terms of the growth in the corresponding control culture without added canavanine (Table XXV). The growth increment on low biotin and 1 mg. of canavanine is greater than that observed on high biotin and 250γ of canavanine. This represents a more than 4 fold increase of resistance due to biotin limitation.

As done here, the effectiveness of low biotin was best demonstrated by adding canavanine to already growing cultures and measuring the inhibition of further growth.

Table XXV

Increased Canavanine Resistance in Biotin Deficiency

Strain 25R11, grown 46 hours before addition of canavanine. Final dry weights taken 47 hours after addition. Mean weight of duplicate pads.

Hours	L-canavanine · $\frac{1}{2}H_2SO_4$ γ/20 ml.	Growth (dry weight)						Relative
		High biotin			Low biotin			
		mg.	abs. Δ	rel. Δ	mg.	abs. Δ	rel. Δ	Low bio. / High bio.
46	0	11.5			5.5			
93	0	61	49.5	100	54.5	49	100	1.00
	100	66.5	55	111	50	44.5	91	.82
	250	26	14.5	29	41.5	36	73.5	2.53
	500	19.5	8	16	31.5	26	53	3.31
	750	18	6.5	13	25	19.5	40	3.08
	1000	16	4.5	9	23.5	18	37	4.11

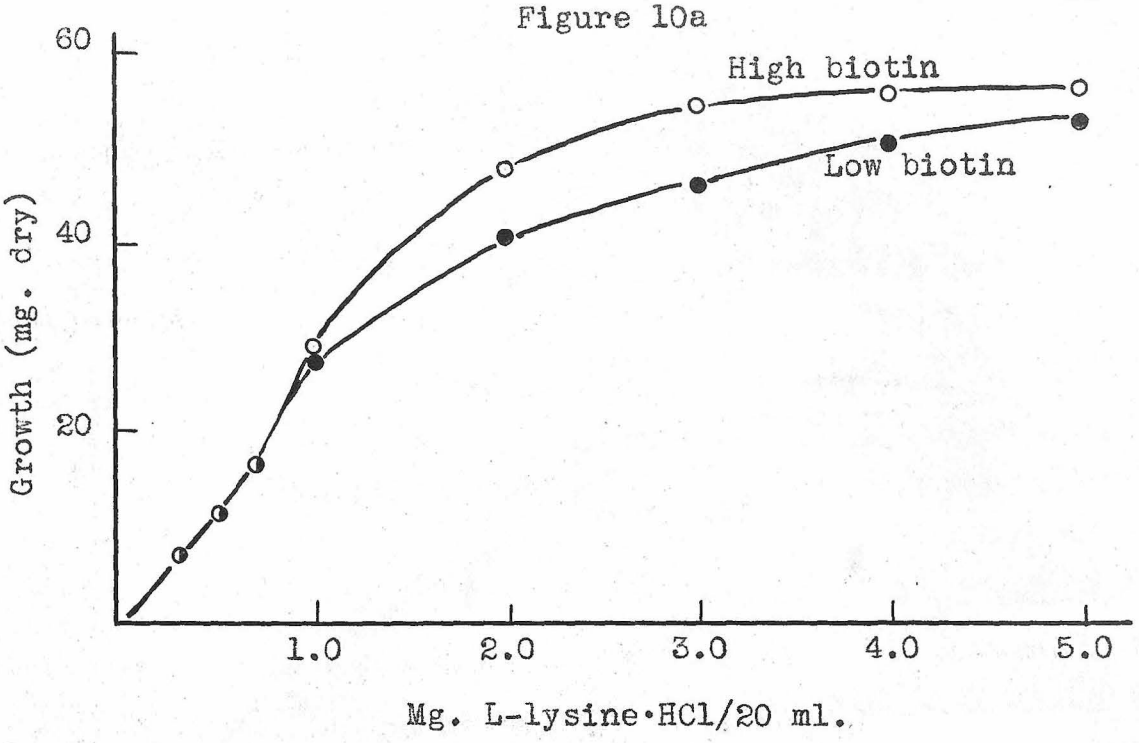
When conidia are inoculated into high and low biotin media containing canavanine, variable results are observed, possibly due to an initial disadvantage caused by low biotin. Essentially the same effect as given in the table has been obtained several times, with a 2 to 4 fold increase of resistance on low biotin. It must be borne in mind, however, that some other detoxification mechanism could also be enhanced by lowering the biotin level.

As a further demonstration that the enzyme is active *in vivo*, the growth responses of three amino acid mutants to varied concentrations of the required amino acid were determined on high and low biotin (Figures 10a, b, and c). Strain 37101 requires L-lysine, and will not grow on D-lysine or on α-keto-ϵ-amino-caproic acid (59). Strain C83, a tryptophaneless mutant, will grow on L- or DL-tryptophane and on indole-pyruvic acid (60). Strain 68604, requiring methionine, is presumed to behave similarly to other methionineless mutants which grow on D- or L-methionine or on α-keto-γ-methylmercaptobutyric acid (4). The only mutant whose response (to different concentrations of its required substrate) is affected by the decrease in biotin concentration is 37101. This can be interpreted as meaning that increased L-oxidase activity decreases the amount of L-isomer available; utilization of a given amino acid is interfered with only where the keto-acid cannot be reconverted to the amino acid.

Comparison of L-oxidases from Medium

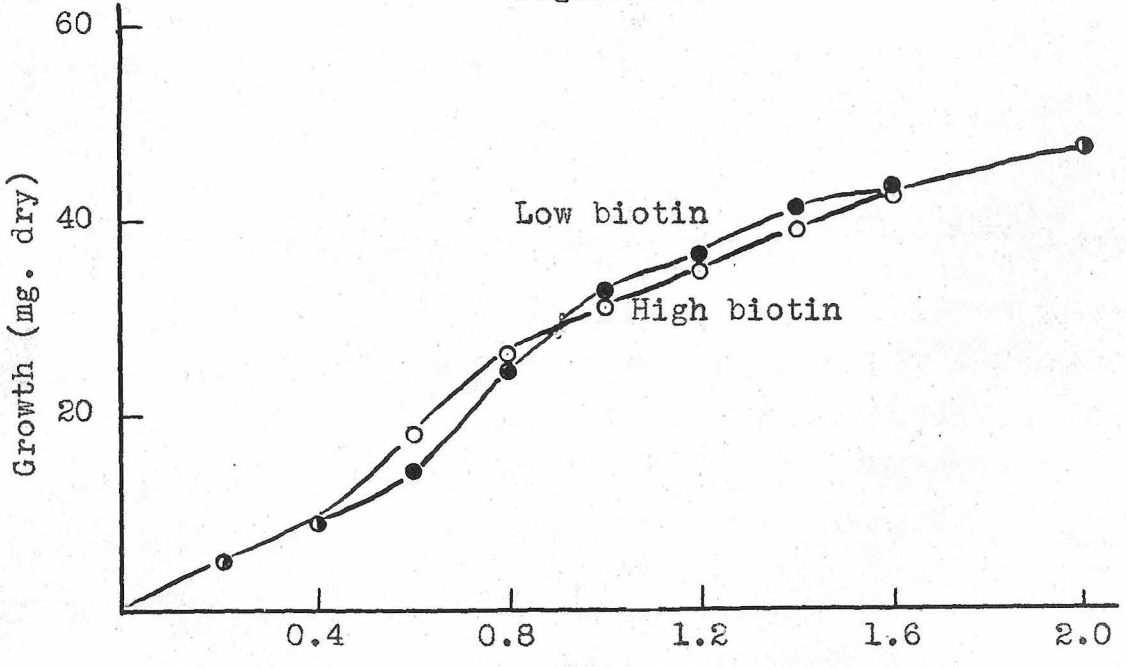
Attempts were made by *in vitro* experiments to find pertinent differences in the activity of the L-oxidase produced in culture medium by sensitive and resistant strains. No significant differences were detected in the activity of enzyme preparations from 4A and 25a on canavanine or other substrates under varied conditions of pH, substrate concentration, enzyme concentration,

Figure 10a



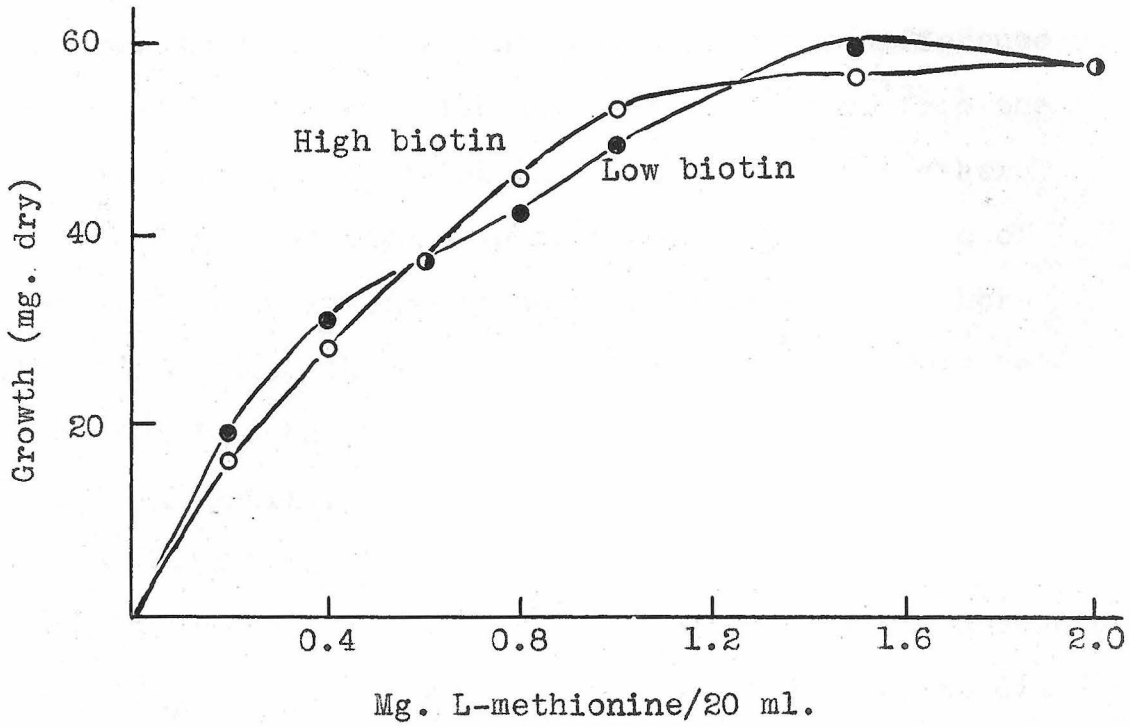
Lysine Response of 37101, 72 hours growth.

Figure 10b



Tryptophane Response of C83, 96 hours growth.

Figure 10c



Methionine Response of 68604, 74 hours growth

and oxygen tension. Nor was there any consistent difference in the amount of L-oxidase activity observed in low biotin extracts. However, it will be seen later that, in a large series of strains, there is a correlation between the level of L-oxidase activity of extracts and canavanine resistance. One very noticeable difference was found in the relative activity of enzymes from the two strains on canavanine and leucine (or some other substrate). In comparable preparations, the ratio of activity on canavanine to that on leucine was higher for a resistant than for a sensitive strain. This held true for 4A when compared to 25a, and for their progeny, 4R10a and 25R11a. However, when 19 of the 22 random ascospore re-isolates from the cross 4A x 25R11a were tested, this difference was found not to segregate with canavanine sensitivity. The re-isolates were tested by using dialyzed extracts of cultures grown on low biotin with casein hydrolysate as sole nitrogen source. Three out of six resistant strains had too low an activity on canavanine, while six out of 13 sensitive strains had too high an activity.

Canavanine Effect in Crude Extracts

When no strain differences were found in the activity on canavanine of the relatively clean enzyme from medium, attention was turned to the effect of canavanine in crude extracts of low biotin cultures. Besides the L-oxidase, such extracts contain most of the free amino

acids of the mycelium, an important part of the intracellular milieu of the enzyme.

Canavanine has been found to inhibit the rate of oxygen uptake by crude extracts of low biotin cultures significantly more than does phenylalanine, leucine, or other substrates. In many cases, the oxygen consumption does not reach the level of the endogenous controls even after prolonged incubation. At other times, oxidation of the added canavanine is ultimately manifested by an increased total oxygen consumption. Such effects are undoubtedly due to competition between canavanine and the endogenous amino acids for the L-oxidase. Models for such competition are provided by the experiments of Table IV (See also Fig. 4), using L-oxidase from medium and prepared mixtures of amino acids.

The effect of canavanine on the respiration of crude low biotin extracts was determined for all 22 of the re-isolates obtained from the cross of 25R11 x 4A. A difference was found between resistant and sensitive strains which parallels their canavanine sensitivity. The effect may be summarized by saying that the more growth-sensitive a strain is to canavanine, the less will the oxygen uptake of a crude extract of that strain be inhibited by the addition of canavanine. Before considering a possible explanation, the data must be discussed in some detail.

The data of Table XXVI are the results of the enzymatic experiments. Inhibition of oxygen uptake by canavanine is given for the time when the endogenous uptake was approximately 50 µl. That the rate of endogenous uptake varied is shown by the different times required to reach the 50 µl. level, and by the figures representing "L-oxidase activity." The resistant strains are different from the sensitive strains in the greater inhibition by canavanine. The mean values for inhibition (0.30 for the resistant strains, 0.11 for the sensitive strains) are significantly different. ($t=5.15$. At 1% level, $t=2.845$. (61))

Great variations in canavanine growth-sensitivity exist among both the sensitive and resistant strains (Tables XXVIIA and XXVIIB). In other tests, some of the resistant strains have shown a higher degree of resistance. The sensitivity of particular strains also varies in different tests. Inoculum size and the age of the culture used for inoculation are undoubtedly important factors in this latter variation. The effect of inoculum size was easily demonstrated. Using a dilute suspension of conidia from strain 11R1, growth was measured after 100 hours on minimal medium alone and with 10γ of canavanine sulfate. With an inoculum of three drops, dry weights were 60 and 0 mg. respectively; four drops, 64.5 and 13; five drops, 70.5 and 26. Critical growth data would depend on the use of inocula identical as to density

Table XXVI

Inhibition of Oxygen Uptake of Crude Extracts by Canavanine

All extracts were of 6 day mycelium grown on 200 ml. low biotin minimal medium. Two ml. of extract contained 0.8 g. FWE. Canavanine--0.25 ml. M/30.

Strain No.	Time Mins.	µl. O_2		Inhibition %/100	Endogenous activity-µl.O_2 in initial 30 mins.
		Endog.	Canav.		
Resistant 11R2	30	51.8	41.2	0.21	52
3	60	55.8	37.2	0.33	30
11	75	54.4	41.6	0.24	20
12	40	57.1	41.0	0.28	42
14	10	46.8	31.8	0.32	131
18	50	54.4	31.3	0.42	35
22	85	55.5	40.5	0.27	18
			Mean	0.30±0.07	47±39
Sensitive 11R1	90	52.4	54.4	-0.04	18
4	90	56.9	55.0	0.03	19
5	90	49.7	47.9	0.04	19
6	75	48.2	42.3	0.12	21
7	95	47.6	41.6	0.13	16
8	75	50.3	47.7	0.05	19
9	60	52.6	44.4	0.16	22
10	60	47.6	43.7	0.08	21
13	50	55.4	43.1	0.22	33
15	90	36.6	36.6	0.00	15
16	70	55.2	47.2	0.15	27
17	95	56.3	46.1	0.18	21
19	30	50.4	36.2	0.28	50
20	40	46.2	40.2	0.13	35
21	85	46.8	41.6	0.11	16
			Mean	0.11±0.08	23±9

Table XXVIIA

Effect of Canavanine on Growth of Resistant Strains

Growth determined after 71-73 hours.

Strain No.	L-canavanine·$\frac{1}{2}H_2SO_4$ γ/20ml.					Inhibition by 100γ
	0	100	200	500	1000	
11R2	55	20	19	8.5	3	0.64
	58	48	53.5			0.17
3	61	19	10.5	14	7	0.69
	64.5	44.5	45			0.31
11	67	21	15	14	17	0.69
	51	29	30.5			0.43
12	54	14	16	16	22	0.74
14	64	18	7	17	13	0.72
18	47.5	30.5	31.5			0.36
22	49.5	34.5	32.5			0.30
					Mean (of means)	0.51±0.17

Table XXVIIB

Effect of Canavanine on Growth of Sensitive Strains

Growth determined after 117-129 hours.

Strain No.	L-canavanine·$\frac{1}{2}H_2SO_4$ γ/20ml.					Inhibition by 10γ
	0	10	25	50	100	
11R1	86	0				1.00
4	89.5	0				1.00
5	90.5	9	0			0.90
6	92.5	13	0			0.86
7	93.5	0				1.00
8	89	24	0			0.73
9	81	58	0			0.28
	61	0				1.00
10	92.5	25.5	0			0.72
13	83	9	30	70.5	0	0.89
	84.5	62	99	3	0	0.27
	79.5	1	3.5	0		0.98
15	84	2	0			0.98
	99.5	33	25	1	0	0.67
16	85.5	20.5	0			0.76
17	85.5	48.5	55	2	0	0.43
19	82.5	0	8.5	0		1.00
	77.5	59.5	54	0		0.25
20	89	40.5	0			0.54
21	82	14	23.5	0		0.83
					Mean (of means)	0.77±0.17

and age. Despite these variations, it was of interest to determine statistically the correlation between the inhibition by canavanine of oxygen uptake in extracts and the growth inhibition by 10γ of canavanine sulfate (sensitive strains only). For the growth inhibition values of Table XXVIIB (average values where more than one determination was made), and the enzyme inhibition values of Table XXVI, a coefficient of correlation of -0.62 is obtained (at 5% level, r=.514; at 1%, r=.641 (61).). The calculated r value is "probably significant." The negative value of r would mean that the strains which are more inhibited in growth show less inhibition of oxygen uptake in extracts. This implies an extension, within the sensitive class, of the conclusion reached by comparing the mean behavior of sensitive and resistant classes. There is not a sufficient number of resistant strains for a similar analysis.

An higher degree of correlation is found when L-oxidase activity (as measured by endogenous oxygen uptake in the first half-hour of incubation) is compared with the degree of inhibition of the endogenous respiration produced by canavanine (sensitive strains only). The correlation coefficient found is 0.74 (p values as before). This also constitutes corroboration of the impression given by comparing mean values for resistant and sensitive strains. The inhibition of oxygen consumption by canavanine would thus be directly related to the amount of

L-oxidase.

Before proceeding to possible interpretations, the conclusions to be drawn from the data presented to this point should be considered. It appears that the sensitive and resistant strains differ significantly in the degree of inhibition by canavanine of oxygen consumption of extracts. The inhibition is greater for the resistant strains. Among the sensitive strains, the degree of inhibition parallels the initial rate of oxygen consumption, and correlates inversely with the sensitivity to canavanine observed in growth experiments.

The question arises: <u>is</u> the rate of oxygen uptake a measure of the L-oxidase activity? Results discussed in an earlier section indicate that it is. In no case has the rate of oxygen consumption of a crude extract with an added L-amino acid appreciably exceeded the maximum rate of oxygen consumption observed in the control extract without added substrate (Cf. Figure 4). This was again demonstrated in the present work with extracts of 11R10 (sensitive) and 11R11 (resistant) using leucine as the substrate. Leucine was oxidized (after long incubation) by extracts of both strains. In other vessels in this experiment, the initial inhibition of oxidation by canavanine was greater in the resistant strain. Toward the end of the run (10 hours) the rate of uptake with canavanine present was increasing for the resistant strain, while the rate for the sensitive strain was constant and

much below the initial level.

Tables XXVI and XXVII show four apparent exceptions to the relation of L-oxidase level to inhibition by canavanine, and to the difference between sensitive and resistant strains. Two resistant strains, 11R11 and 11R22, have L-oxidase activities similar to those of sensitive strains, yet the extracts show inhibition by canavanine characteristic of the resistant strains. Two sensitive strains, 11R13 and 11R19, show L-oxidase activity and inhibition by canavanine characteristic of the resistant strains. Strains 11R13 and 11R19 are atypical in other respects. Their growth response to canavanine shows stimulation by intermediate concentrations (Table XXVIIB). Total yields of L-oxidase produced on low biotin-casein hydrolysate medium (Table XXII) are high. These differences suggest a different degree of "adaptivity" of the two strains, and a tentative explanation which preserves the validity of the conclusions. The canavanine sensitivities were determined on high biotin medium, where L-oxidase activities are low. If these two strains are abnormally "sensitive" to amino acids in the adaptive formation of the enzyme, growth in low biotin minimal might produce a greater increase of enzyme than occurs with the other strains. Since there appears to be a correlation between amount of activity and the degree of inhibition in extracts, the deviation from the mean inhibition would be a result of the higher activity. On this basis, 11R13 and 11R19

would be expected to show a greater increase of canavanine resistance by growth on low biotin over high biotin than would other sensitive strains. However, when tested by the delayed addition of canavanine (as in Table XXV) these two strains showed nearly equal resistance on high and low biotin. Two control strains, 25R11 and 11R10, showed 80% more resistance on low biotin.

An alternative explanation, unexplored experimentally, is similarly based on the adaptive "sensitivity" of these strains. It is possible that increased L-oxidase activity can be elicited by canavanine (or other amino acids) even on high biotin medium if a strain is sufficiently sensitive. Such an idea might find support in the apparent stimulation of growth of strains 11R13 and 11R19 by intermediate concentrations of canavanine.

Discussion

The experiments reported above show that the L-amino acid oxidase is one factor involved in the detoxification of canavanine. This makes an interpretation of several observations necessary, especially of those made with crude extracts of the 22 re-isolated strains. A fundamental problem must also be taken into account. The L-oxidase has been demonstrated in all sensitive, as well as resistant, strains tested. The non-toxicity of the product of L-oxidase action on canavanine has been demonstrated. The basic question to be answered is, therefore: why is this potential mechanism of detoxifica-

tion apparently not operative in sensitive strains of the mold? In addition, what is it that segregates with canavanine sensitivity which causes a greater inhibition of oxygen uptake (by canavanine) in extracts of resistant strains? Can the exceptional strains be accounted for in any scheme based on the present observations?

The most direct explanation postulates a difference in the nature of the L-oxidase of sensitive and resistant strains. Its affinity for canavanine may be modified in such a way in the sensitive strains that, in the presence of the free amino acids of the mycelium, canavanine is attacked much more slowly than in the resistant strains. In this case, greater inhibition of oxygen uptake in extracts, as for the resistant strains, would indicate that canavanine was being oxidized in preference to other substrates. The difference would not have to be an absolute one, since it is known that sensitive strains will eventually grow on moderate concentrations of canavanine (2). Moreover, the delayed addition experiments reported above show that started cultures of sensitive strains will grow on concentrations of canavanine much higher than those which prevent initiation of growth.

Two experimental tests of the present hypothesis are proposed. Firstly, by detailed chromatographic or other studies it should be possible to determine if canavanine were being oxidized preferentially in extracts of resistant strains and more rapidly than in corresponding

extracts of sensitive strains. Secondly, it should be possible to determine if the qualitative amino acid distribution in extracts is a contributing factor in producing the difference in inhibition of endogenous respiration. This could be done by reconstitution tests with boiled extracts and dialyzed extracts from sensitive and resistant strains. The crucial test would be the comparison of the effect of canavanine on oxidation of the homologous and heterologous boiled extracts by each dialyzed extract.

Genetic control of canavanine resistance was demonstrated by Horowitz and Srb (2). Out of 18 asci dissected, 13 indicated the segregation of resistance as a single gene effect, although the remaining 5 asci "could not be so simply interpreted." This is suggestive of the presence of genetic or other modifiers of the primary effect. Subsequent work by Srb and his students (62) has always shown the results expected of a single gene effect. Since details of the crosses are not available, it may be possible that the modifying genes were eliminated by the choice of parents. Teas (51) suggests that the inability of the resistant mutant strain 51504 to use canavanine as a substitute for homoserine may be due to its resistance being of a different sort from that of 4A (relative to 25a). The presence of a second gene was also suggested in the present work by the isolation of 25R9, partially sensitive, from the cross 4A x 25a.

The ascus from which it was derived showed this distribution by spore pairs:

sensitive;	partially sensitive;	resistant;	sensitive.
25R8	25R9	25R10	25R11

Further crosses to test for genetic or other factors producing this result have not been done, and the cross 4A x 25R11a resulted in no dissectable asci in two attempts. The present enzymatic and growth data could also be interpreted as indicating the presence of genetic modifiers, notably in respect to the exceptional sensitive and resistant strains.

The biochemical, genetic, and growth data indicate that at least two factors are involved in the determination of canavanine sensitivity. They must be assumed to vary independently, at least within limits; nor is it possible to point to either as the primary genetically controlled factor. It is clear that the L-oxidase is involved in the detoxification of canavanine. The growth experiments with high and low biotin show that the specific L-oxidase activity of mycelium is one important factor. In addition to this quantitative factor, a qualitative difference in the activity on canavanine of the L-oxidase of sensitive and resistant strains is apparent from the enzymatic experiments. A further qualitative difference, in the distribution pattern of free amino acids in the mycelium of sensitive and resistant strains, may contribute to, or even produce, this apparent enzymatic difference.

References

1. Bender, A. E., Krebs, H. A., and Horowitz, N. H., Biochem. J., 45, xxi (1949).

2. Horowitz, N. H., and Srb, A. M., J. Biol. Chem., 174, 371 (1948).

3. Bender, A. E., and Krebs, H. A., Biochem. J., 46, 210 (1950).

4. Horowitz, N. H., J. Biol. Chem., 154, 141 (1944).

5. Zeller, E. A., Advances in Enzymol., 8, 459 (1948).

6. Krebs, H. A., in The Enzymes (Sumner, J. B., and Myrback, K., eds.), II, Pt. 1, 499 (Academic Press, 1951).

7. Krebs, H. A., Biochem. Soc. Symp., 1, 2 (1948).

8. Thayer, P. S., and Horowitz, N. H., J. Biol. Chem., in press (1951).

9. Burton, K., Biochem. J., in press (1951).

10. Westergaard, M., and Mitchell, H. K., Am. J. Bot., 34, 573 (1947).

11. Horowitz, N. H., J. Biol. Chem., 171, 255 (1947).

12. Kearney, E. B., and Singer, T. P., Arch. Biochem., 21, 242 (1949).

13. Friedemann, T. E., and Haugen, G. E., J. Biol. Chem., 147, 415 (1943).

14. Adamson, D. W., J. Chem. Soc., (1939), 1564.

15. Akabori, S., and Numano, S., Bull Chem. Soc. Japan, 11, 214 (1936).

16. Keilin, D., and Hartree, E. F., Biochem. J., 39, 293 (1945).

17. Singer, T. P., and Kearney, E. B., Arch. Biochem., 29, 190 (1950).

18. Haldane, J. B. S., Enzymes (Longmans, Green, 1930).

19. Lineweaver, H., and Burk, D., J. Am. Chem. Soc., 56, 658 (1934).

20. Edlbacher, S., and Wiss, O., Helv. Chim. Acta, 28, 797 (1945).

21. Cohn, E. J., and Edsall, J. T., Proteins, Amino Acids and Peptides (Reinhold, 1943).

22. Borek, E., and Clarke, H. T., J. Biol. Chem., 125, 479 (1938).

23. Windsor, E., Doctoral thesis, California Institute of Technology (1950).

24. Catch, J. R., and Jones, T. S. G., Biochem. J., 42, lii (1948).

25. Hughes, D. E., Brit. J. Exp. Path., 32, 97 (1951).

26. Horowitz, N. H., J. Biol. Chem., 162, 413 (1946).

27. Gomori, G., Proc. Soc. Exp. Biol. and Med., 68, 354 (1948).

28. Gomori, G., Proc. Soc. Exp. Biol. and Med., 62, 33 (1946).

29. Warburg, O., and Christian, W., Biochem. Z., 298, 150 (1938).

30. Moore, S., and Stein, W. H., J. Biol. Chem., 176, 367 (1948).

31. Fling, M., and Horowitz, N. H., J. Biol. Chem., 190, 277 (1951).

32. Ryan, F. J., Beadle, G. W., and Tatum, E. L., Am. J. Bot., 30, 784 (1943).

33. Hodson, A. Z., J. Biol. Chem., 157, 383 (1945).

34. Winzler, R. J., Burk, D., and du Vigneaud, V., Arch. Biochem., 5, 25 (1944).

35. Snell, E. E., and Wright, L. D., Ann. Rev. Biochem., 19, 277 (Annual Reviews, 1950).

36. Hodson, A. Z., J. Biol. Chem., 179, 49 (1949).

37. Bhagvat, K., and Devi, P., Biochem. J., 45, 32 (1949).

38. Monod, J., Growth, 11, 223 (1947).

39. Spiegelman, S., in The Enzymes (Sumner, J. B., and Myrback, K., eds.), I, Pt. 1, 267 (Academic Press, 1950).

40. Luria, S. E., and Delbruck, M., Genetics, 28, 491 (1943).

41. Block, R. J., and Bolling, D., The Determination of the Amino Acids, rev. ed. (S. S. Publishing Co.,1948).

42. Hungate, F. P., Doctoral thesis, Stanford University (1946).

43. Fling, M., unpublished observations.

44. Shen, S. C., Doctoral thesis, California Institute of Technology (1950).

45. Horowitz, N. H., Growth Symposium, in press (1951).

46. Nason, A., Kaplan, N. O., and Colowick, S. P., J. Biol. Chem., 188, 397 (1951).

47. Nason, A., Federation Proc., 10, 228 (1951).

48. Blanchard, M. L., Korkes, S., del Campillo, A., and Ochoa, S., J. Biol. Chem., 187, 875 (1950).

49. Gulland, J. M., and Morris, C. J. O. R., J. Chem. Soc., (1935), 763.

50. Kitagawa, M., and Takani, A., J. Biochem., Japan, 23, 181 (1936).

51. Teas, H. J., J. Biol. Chem., 190, 369 (1951).

52. Volcani, B. E., and Snell, E. E., J. Biol. Chem., 174, 893 (1948).

53. Bonner, J., Am. J. Bot., 36, 323 (1949).

54. Bonner, J., Am. J. Bot., 36, 429 (1949).

55. Wright, J. E., and Srb, A. M., Bot. Gazette, 112, 52 (1950).

56. Teas, H. J., Doctoral thesis, California Institute of Technology (1947).

57. Damodaran, M., and Narayanan, K. G. A., Biochem. J., 34, 1449 (1940).

58. Kitagawa, M., and Tsukamoto, J., J. Biochem., Japan, 26, 373 (1937).

59. Good, N., Heilbronner, R., and Mitchell, H. K., Arch. Biochem., 28, 464 (1950).

60. Mitchell, H. K., unpublished observations.

61. Snedecor, G. W., Statistical Methods (Iowa State College Press, 1940).

62. Srb, A. M., personal communication to Dr. N. H. Horowitz.

Appendix

Minimal Medium Used for *Neurospora* (11)

NH_4 Tartrate	5.0 g.
NH_4NO_3	1.0 "
KH_2PO_4	1.0 "
$MgSO_4 \cdot 7H_2O$	0.5 "
NaCl	0.1 "
CaCl	0.1 "
$Na_2B_4O_7 \cdot 10H_2O$	0.088 mg.
$CuCl_2 \cdot 2H_2O$	0.268 "
$FeCl_3 \cdot 6H_2O$	0.978 "
$MnCl_2 \cdot 4H_2O$	0.072 "
$Na_2MoO_4 \cdot 2H_2O$	0.050 "
$ZnCl_2$	4.180 "
Sucrose	20.0 g.
Biotin	5.0 γ
H_2O	1.0 liter